THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 241, No. 14, Issue of July 25, pp. 3365-3372, 1966
Printed in U.S.A.

Regulatory Enzymes of Aromatic Amino Acid Biosynthesis in *Bacillus subtilis*

I. PURIFICATION AND PROPERTIES OF 3-DEOXY-D-*ARABINO*-HEPTULOSONATE 7-PHOSPHATE SYNTHETASE*

(Received for publication, February 3, 1966)

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SUMMARY

1. The 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthetase of *Bacillus subtilis* has been purified 77-fold (more than 500-fold over the activity in crude extracts of wild-type strains grown in a minimal salts medium).

2. The following points support the thesis that DAHP synthetase is a single enzyme: (*a*) The sensitivity of the enzyme to two inhibitors is constant throughout a 77-fold purification. (*b*) Prephenate and chorismate independently inhibit the enzyme activity 80% at saturating inhibitor concentrations. (*c*) Fractionation procedures yield nonseparable DAHP synthetase activities which are similar in properties such as storage stability, thermostability, inactivation by urea or low pH, and in experimental values of kinetic constants of enzyme velocity. (*d*) The logic for one DAHP synthetase as the initial metabolic control point for pathway-wide regulation is consistent with efficient control by the feedback inhibitors, chorismate and prephenate. (*e*) Auxotrophs which are deficient for DAHP synthetase occur as single step revertible mutations.

3. Modifications in the assay for DAHP synthetase activity in *B. subtilis* are described.

4. Partially purified preparations of enzyme have a pH optimum of 6.5, a pH stability optimum of 6.9, and a temperature optimum of 55°. An activation energy of 9,100 cal per mole was calculated. The enzyme is activated 2-fold by low concentrations of *p*-chloromercuribenzoate and iodoacetate and inhibited by concentrations of 2-mercaptoethanol exceeding 10^{-5} M. Molecular weights of 134,000 and 129,000 were determined by gel filtration and sucrose density gradient techniques, respectively.

Enzymological comparisons of control mechanisms in different organisms have revealed the remarkable versatility which characterizes the control of the activity of enzyme systems catalyzing identical sequences of biochemical reactions. A system of individually controlled isoenzymes represents one of several types of control mechanisms known to regulate branched metabolic pathways. In particular, the multibranched aromatic acid biosynthetic pathway in *Escherichia coli* (strain W) is controlled by specific allosteric interactions of tyrosine, phenylalanine, or tryptophan with three cognate isoenzymes (1, 2). The studies reported in this paper originated with an investigation of the control of the activity of the first enzyme of this pathway in *Bacillus subtilis* and led to preliminary evidence that its 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthetase[1] was a single protein. Subsequent confirmatory data which are detailed in this paper proved compatible with a new pattern of regulation by a highly integrated system of control via four regulatory enzymes (3, 4). While this latter description of the inhibition of DAHP[2] synthetase by prephenate and chorismate rather than by amino acid end products mitigates against a system of isoenzyme control comparable to that of *E. coli*, there remained some possibility for the existence of two isoenzymes, each specifically controlled by one of the inhibiting intermediary metabolites. This possibility was thoroughly examined as one experimental approach to the study of the relative significance of the two feedback effectors *in vivo*. The purpose of this communication is to cite the evidence that only one DAHP synthetase protein is synthe-

* This project was supported in part by Research Grants GM 09848-03, 1-F2-AI-22,778-01, and 5 T1 GM-511-05 from the National Institutes of Health, United States Public Health Service, and by the State of Washington Initiative 171 Funds for Biological and Medical Research.

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[1] The biosynthetic reaction is a condensation of P-enolpyruvate and D-erythrose-4-P to form orthophosphate and 3-deoxy-D-*arabino*-heptulosonate 7-phosphate. The latter designation has been used in accordance with both the Carbohydrate Committee rules and Chemical Abstracts usage since 1960. However, the Enzyme Commission has adopted a different systemic name (7-phospho-2-oxo-3-deoxy-D-*arabino*-heptonate D-erythrose-4-phosphate-lyase (pyruvate phosphorylating), EC 4.1.2.15) and recommended the use of the trivial name, phospho-2-keto-3-deoxyheptonate aldolase. In spite of the fact that the reaction catalyzed by 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthetase is irreversible, the E.C. name is based upon the reverse reaction. It is also clear that the reaction is not an aldol condensation. The E.C. name is further inappropriate because of the implication that pyruvate would be phosphorylated, even though pyruvate is not a participant in the reaction.

[2] The abbreviations used are: DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; TEAE, "triethylaminoethyl" ($-C_2H_4-N^+-(C_2H_5)_3$).

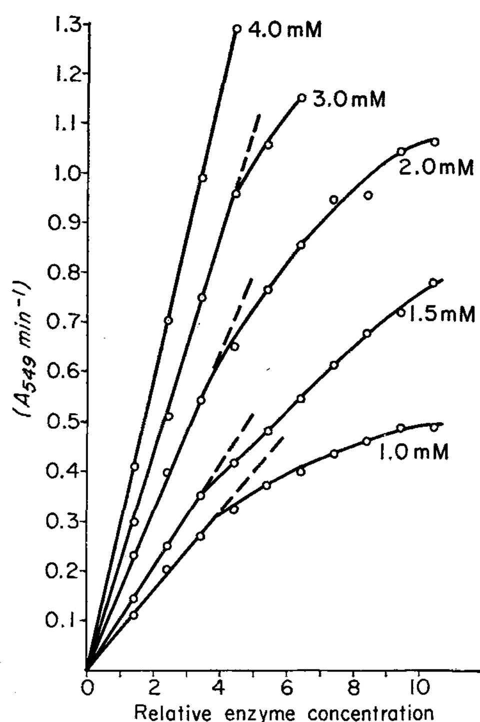

FIG. 1. Variation of enzyme activity as a function of enzyme concentration at various equal substrate concentrations of erythrose-4-P and P-enolpyruvate (concentrations indicated on each *curve*). A crude extract from strain SB 491, prepared by sonic disruption, was used (41 μg per ml, final protein concentration corresponding to 1 on the *abscissa*). Reaction tubes were incubated for 10 min at 37°. Substrate solutions were freshly prepared.

sized by *B. subtilis;* this enzyme is also characterized and experimental details relevant to its purification and assay are reported.

EXPERIMENTAL PROCEDURE

Materials and Methods

Bacterial Strains and Extract Preparation—Strains of *B. subtilis*, growth conditions, media, and the preparation of extracts from these strains were described by Jensen and Nester (3).

DAHP Synthetase Assay—DAHP synthetase was assayed by the chemical determination of DAHP produced by the enzymatic condensation of erythrose-4-P and P-enolpyruvate (5). The reaction mixture contained 20 μmoles of phosphate buffer, pH 6.5; 0.4 μmole of erythrose-4-P (Calbiochem); and 0.4 μmole of P-enolpyruvate (Sigma) in a total volume of 0.20 ml. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 mμmole of DAHP per min. The amount of DAHP was evaluated with the use of the value of 4.5×10^4 for the molar extinction coefficient at 549 mμ. The reaction was initiated by combining 0.05 ml of extract with 0.15 ml of reaction mixture (at 0° and removing the tubes to a 37° bath); it was terminated with 0.04 ml of 20% trichloracetic acid. Protein was sedimented by centrifugation and 0.10 ml of the supernatant was added to 0.10 ml of 0.025 M periodate in 0.125 N H_2SO_4 and incubated at 37° for 30 min. The subsequent addition of 0.20 ml of 2% sodium arsenite in 0.5 N HCl was followed by the addition of 0.80 ml of 0.3% thiobarbituric acid (5). The color was developed by heating at 100° in marble-capped tubes for 10 min and the absorbance was read at 549 mμ in a Beckman model DU spectrophotometer. The turbidity correction described by Doy and Brown (6) was circumvented by maintaining the reaction mixture at 55° following the color development until the absorbance was measured. The concentration of substrates originally used (5) in the reaction mixture was increased in order to expand the range where enzyme activity is proportional to reaction time and to protein concentration. No substrate inhibition occurred when erythrose-4-P and P-enolpyruvate were varied up to concentrations of 4 mM. A typical family of activity curves obtained at different concentrations of substrates as a function of enzyme concentration is shown in Fig. 1. There is a linear proportionality between enzyme concentration and enzyme activity up to the utilization of about 30% of the substrates, and the proportion of substrates utilized by the undiluted extract of Fig. 1 in 10 min is 45 to 50%. The reaction mixture of erythrose-4-P and P-enolpyruvate in phosphate buffer was as stable at −20° as was the storage of individual substrate solutions. The preparation of erythrose-4-P by the hydrolysis of dicyclohexylammonium D-erythrose 4-phosphate dimethylacetal hydrate (7) resulted in somewhat variable concentrations of this substrate. Because of this difficulty and the lability of this compound, and because the rate of reaction was not zero order with respect to substrates (see Fig. 1), it was desirable to measure enzyme activities against a reference standard in order to make quantitative comparisons of enzyme activities that were assayed with different batches of substrate. The *B. subtilis* enzyme is sufficiently stable that aliquots of crude extracts stored at −20° lose no activity for at least 6 months, and these aliquots were used as reference standards. Activities were corrected by the use of a factor equivalent to the ratio of the activity of the reference standard initially determined to the activity of the reference standard (in duplicate) measured in any given experiment. This procedure also corrected for errors arising from the variations in the duration and temperature of the enzyme reaction as well as differences influencing the oxidation of DAHP by periodate in the chemical assay (6). The correction factor was generally between 1.0 and 1.5. All reagents used in enzyme analyses were made up in glass-distilled water.

Gel Filtration for Molecular Weight Determination—Sephadex G-100 and G-200 gel columns were prepared and calibrated with cytochrome *c* and bovine serum albumin (containing serum albumin dimer) as internal standards according to the procedure of Andrews (8). The proteins used as internal standards were obtained from Sigma. The dimensions of the column were 2.4 × 50 cm and the flow rate was adjusted to 28 ml per hour. Protein solutions were applied to the top of the column in a volume of 1.0 ml by increasing the density with sucrose (2 mg) and layering the sample under the buffer above the column bed.

Sucrose Density Gradients—Linear gradients of sucrose from 6 to 25% containing 0.04 M phosphate, pH 6.8, were prepared in volumes of 4.7 ml. Internal standards and extract proteins were layered on each sucrose density gradient in a volume of 0.2 ml. These were centrifuged for 20 hours at 10° and 38,000 rpm in the SW 39 rotor of the model L preparative ultracentrifuge (Spinco). Forty 2-drop fractions were collected after piercing the tube with a small diameter needle. The fractions were dialyzed (0.04 M phosphate, pH 6.8, in 0.10 M KCl) as a preliminary step to the assay of DAHP synthetase activity since sucrose interferes with the chemical assay for DAHP. Molecular weights were estimated according to the method of Martin and Ames (9). Reference standards used to calculate the molecular weight were

bovine liver catalase, twice crystallized (Sigma); *E. coli* alkaline phosphatase (Worthington); and human hemoglobin, twice crystallized (Calbiochem). Catalase was assayed by adding 1.0 ml of appropriately diluted sample to 2.0 ml of substrate solution (0.60 ml of 30% H_2O_2 diluted to 100 ml with 0.05 M phosphate buffer, pH 7.0) and recording the rate of decrease in absorbance at 240 mμ. Alkaline phosphatase was assayed by the method of Torriani (10). Human hemoglobin was estimated by its absorbance at 410 mμ.

Dialysis—Crude extracts were stable to dialysis. Partially purified preparations such as those recovered from Purification Procedures 1 and 2 were somewhat unstable to prolonged dialysis, losing about 30% of their original activity after 16 hours at 4° in 0.04 M potassium phosphate in 0.10 M KCl, pH 6.8. KCl appeared to stabilize DAHP synthetase activity and was used routinely at this concentration; the reaction velocity of DAHP synthetase did not vary appreciably as a function of ionic strength.

Whole Cell Enzyme Assays—Cultures (10.0 ml) in the logarithmic phase of growth were centrifuged and washed once in 5.0 ml of minimal salts medium (3). They were concentrated by resuspension in 0.5 ml of minimal salts medium, treated with 0.1 ml toluene for 5 min at 37°, and assayed for DAHP synthetase activity as described above.

RESULTS

Partial Purification

All fractionation procedures were done with extracts of strains which were derepressed for DAHP synthetase in order to avoid the possible preferential repression of the synthesis of one or more isoenzyme species.

Growth of Cells and Extract Preparation—The purification regimen shown in Table I was carried out with extracts made from cultures of SB 167, an auxotroph which grows on shikimic acid-supplemented media (3). This strain, grown in a minimal salts medium, supplemented with shikimic acid (30 μg per ml) displays characteristically high specific activities ranging between values of 140 and 170 units per mg of protein. This magnitude of derepression represents about a 7-fold elevation in DAHP synthetase activity compared to the enzyme activity of wild type strains grown in an unsupplemented minimal salts medium. The constitution of the minimal medium and other supplements, conditions of growth, and preparation of extracts by sonic disruption have been detailed previously (3). Fraction I of Table I is the dialyzed supernatant of the crude extract after centrifugation at 25,000 × *g* for 30 min.

Protamine Sulfate Treatment—A solution of protamine sulfate, 20 mg per ml, was adjusted to a pH of 6.5 with 1 N NH_4OH and added to Fraction I slowly with continuous stirring (0.5 ml/70 mg of protein). The resulting suspension was stirred for 15 min at 0° and centrifuged at 15,000 × *g* for 15 min. An additional clarification (25,000 × *g* for 30 min) was necessary.

Heat Treatment—Aliquots of 3 ml of the supernatant fluid from the protamine treatment (Fraction II) were distributed into small tubes and brought to 37° in a water bath. The tubes were transferred to a 55° water bath and chilled in ice after 4 min. The precipitate was removed after centrifugation at 15,000 × *g* for 15 min, and the supernatant was frozen in a Dry Ice-alcohol bath and stored overnight at −20°. When this was thawed much of the protein was insoluble. The supernatant (Fraction III) remaining after another centrifugation at 15,000 × *g* for 15 min was retained.

Sephadex G-100 Fractionation—A column (75 × 3.2 cm) was prepared according to the procedure of Andrews (8) and equilibrated with 0.04 M potassium phosphate buffer containing 0.1 M KCl, pH 6.8. Then 10 ml of Fraction III were applied to the top of the column and eluted at 4° in 5-ml fractions with the above phosphate-KCl at a flow rate of 18 ml per hour. This procedure was repeated with an additional 15 ml of Fraction III and the 11 fractions of peak activity from the two column runs were pooled (Fraction IV). DAHP synthetase was eluted near the front of the protein eluate.

Ammonium Sulfate Fractionation—Fractionation of Sephadex eluates by $(NH_4)_2SO_4$ was accomplished by the use of sufficient saturated ammonium sulfate at pH 6.8 containing 0.1 mM EDTA to give a final saturation of 50%. The precipitate was removed by centrifugation and more saturated $(NH_4)_2SO_4$ was added to bring the level of saturation to 55%. The precipitate from this fraction was removed by centrifugation and a final fraction precipitating between 55 to 65% saturation was obtained. The latter fraction, resuspended in 0.04 M potassium phosphate buffer at pH 6.8 (Fraction V), contained most of the enzyme activity.

Diethylaminoethyl Cellulose Chromatography—Fraction V was dialyzed against 1000 volumes of 0.04 M phosphate buffer, pH 6.8 (3 hours, two changes of buffer), after which it was applied to the top of a column, 2.1 × 25.5 cm, of DEAE-cellulose which had been equilibrated against the same buffer and capped with a 3-cm layer of Sephadex G-25. The enzyme was eluted with a linear salt gradient of KCl, 0.05 M to 0.50 M in 0.04 M potassium phosphate buffer, pH 6.8. Fractions of 2.0 ml were collected at a flow rate of 12.1 ml per hour and the three tubes containing peak enzyme activities were pooled (Fraction VI). The enzyme was eluted at a KCl concentration of 0.25 to 0.30 M.

TABLE I

Partial purification of B. subtilis DAHP synthetase

Fraction	Volume	Concentration	Total activity	Protein concentration	Specific activity	Yield	Purification[a]
	ml	*units/ml*[b]	*units*	*mg/ml*	*units/mg protein*	%	
I. Crude soluble sonic extract	25.0	4,131	103,275	27.54	150	100	1.0
II. Protamine sulfate supernatant	27.0	4,090	110,430	26.91	152	107	1.0
III. Heat-stable supernatant	26.5	4,061	107,616	11.84	343	104	2.3
IV. Sephadex G-100 eluates	55.0	1,107	60,885	0.71	1,559	59	10.4
V. Ammonium sulfate (55 to 65%)	3.0	13,966	41,898	3.58	3,901	41	26.0
VI. DEAE-cellulose eluates	6.0	2,075	12,450	0.18	11,528	12	76.9

[a] Initial 7-fold purification due to derepression of enzyme, see text.

[b] One unit equals an activity equivalent to the formation of 1 mμmole of DAHP per min.

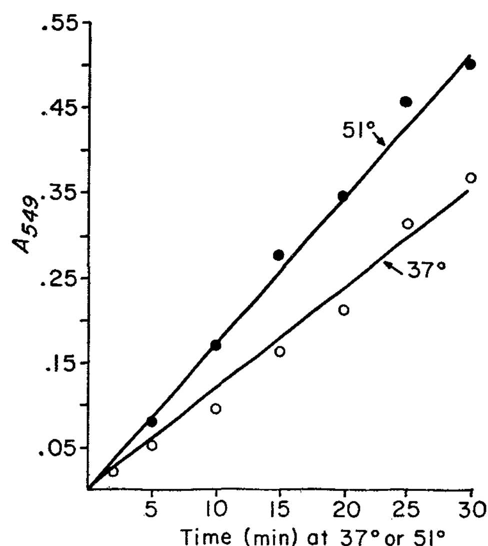

FIG. 2. Plot of velocity (expressed as absorbance at 549 mμ) as a function of time at two temperatures. The extract was purified 2.1-fold from strain WB 698b, a derepressed mutant, by the use of the procedures through Step III (Table I). Cells were disrupted by lysozyme-DNase treatment and a final protein concentration of 125 μg per ml was used in the reaction.

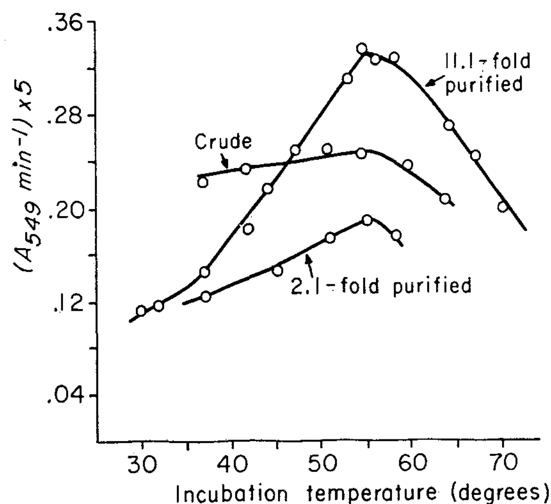

FIG. 3. Dependence of reaction velocity upon the reaction temperature. Each experimental point is an average of three independent determinations. The extracts, crude, 2.1-fold purified (heat treatment), and 11.1-fold purified (Procedure 1) were made from strain SB 167. The crude extract had a specific activity of 161 units per mg. Incubation interval, 5 min.

Other Purification Procedures

The fractionation procedure outlined in Table I results in a 77-fold purification and exceeds the activity of wild type extracts by a factor of more than 500. Fraction VI is unstable and loses about 70% of its activity in 3 days. Further attempts to concentrate enzyme activity by ammonium sulfate precipitation led to an abrupt loss in activity. Although the procedure of Table I resulted in a higher degree of purification, simpler procedures resulting in preparations purified between 10- and 50-fold were much more stable, and, in practice, these were the preparations used in most of the experiments described in this communication. Three variations in purification procedure were useful in this connection.

Procedure 1—The exact steps described in Table I through the preparation of Fraction IV were followed. Sephadex eluates could be stored at −20° for at least a month without significant loss in activity.

Procedure 2—Fraction III of Table I could be satisfactorily fractionated by the addition of solid ammonium sulfate at 0°. The activity was usually broadly distributed between fractions taken between 50 and 75% of ammonium sulfate saturation. The activity profiles varied to the extent that peak activity sometimes occurred in the 50 to 55% of saturation cut and sometimes in the 55 to 65% of saturation cut. The $(NH_4)_2SO_4$ fraction of peak activity yielded an increase in specific activity of about 5 with respect to Fraction III. Thus, salt fractionation of Fraction III produces about twice the purification then obtained with salt fractionation of Fraction IV.

Procedure 3—Fraction II (see Table I) was eluted from DEAE-cellulose as described above. Linear gradients of either phosphate buffer or of KCl (containing 0.04 M phosphate buffer, pH 6.8) gave comparable results. This was the most effective purification step, producing an increase of activity over Fraction II on the order of 50-fold. Like Fraction VI, this preparation lost activity as a result of ammonium sulfate precipitation and tended to be unstable, losing 70% of its activity after 1 week at 4°.

Properties of DAHP Synthetase

Reaction Kinetics—DAHP synthetase displays uncomplicated reaction kinetics under the described conditions of assay. Fig. 1 shows that the reaction rate is a linear function of protein concentration within a limited range of enzyme concentration. No disproportional loss of enzyme activity occurred with the dilution of extracts. For any one concentration of enzyme, the activity is proportional to the concentration of substrates. Because of the relatively high K_m for erythrose-4-P, the enzyme was not saturated with this substrate up to concentrations of 4 mM. Since it was not practical to use higher concentrations of erythrose-4-P, a level of 2 mM was chosen for enzyme assay. Fig. 2 indicates that the linearity of the reaction rate is maintained throughout a fairly wide range of temperature for at least 30 min. An increase in rate of activity at higher reaction temperatures is correlated with the degree of purification of the enzyme. Data obtained from three extracts varying in relative degree of purification is shown in Fig. 3 where a comparison is made of the shape of activity curves plotted as a function of reaction temperature. If one compares initial velocities at reaction temperatures of 37 and 51° the activity ratios corresponding to crude, 2.1-fold purified, and 11.1-fold purified preparations are 1.1, 1.4, and 2.0, respectively. Purification Procedures 1, 2, and 3 (and most preparations purified more than 5-fold) consistently yielded preparations in which activity-temperature curves approximated that of the 11.1-fold purified extract shown in Fig. 3. The basis of this effect is unknown. Since the reaction rates were linear functions of time throughout this temperature range (see Fig. 2) independently of degree of purification, the effect cannot be associated with a partial inactivation of the enzyme. Perhaps some form of temperature-dependent inhibition occurs in crude extracts. (The inhibition of the enzyme by prephenate and chorismate is extremely temperature dependent, but this effect does not vary with purification (11).) Enzyme preparations purified more than 10-fold display a temperature optimum of 55° for reaction times of at least 5-min duration. Fig. 4 is a standard Arrhenius plot the logarithm of enzyme velocity plotted against the reciprocal of

absolute temperature. The activation energy calculated from the negative slope $-E/R$ has a value of 9100 cal per mole. Below temperatures where thermal inactivation of enzyme occurred, no irregularities of slope were observed. (The value of V_{max} was determined only at 37° and it is assumed that the enzyme was saturated in substrates at all other temperatures.)

Effect of pH—The data relevant to the influence of hydrogen ion concentration upon both enzyme stability and enzyme activity appear in Fig. 5. DAHP synthetase activity increases abruptly with increasing pH to an optimal pH of 6.5. Further increases in pH to pH 7.5 result in a gradual decrease in activity of about 15%. The enzyme is quite stable at 4° in the pH range 6.0 to 8.0 for durations of at least 18 hours; a broad stability curve characterizes the latter pH range with activity varying less than 2-fold. The enzyme is stable at hydrogen ion concentrations permitting only low reaction rates (*e.g.* pH 6.0). The optimal pH for maintenance of enzyme stability was 6.8 to 7.0. For this reason purification procedures were carried out at pH 6.8 whenever possible; for uniformity it was convenient to assay the enzyme activity at this same pH, even though this pH is slightly higher than the optimal pH of 6.5 (the activity at pH 6.8 is about 94% of that measured at pH 6.5). The dependence of enzyme activity upon pH did not appear to differ significantly between crude and purified preparations.

Thermostability—Elevated temperatures inactivate DAHP synthetase with first order kinetics. One milligram of protein in a volume of 1.0 ml, prewarmed to 37°, lost 50% of the original enzyme activity in 4 min at 66°. Similar inactivation curves were observed in comparing numerous preparations of varying specific activity and degree of purification.

Inhibitors and Activators—Perhaps the most dramatic difference between the DAHP synthetases of *E. coli* and *B. subtilis* is their response to thiol reagents. The concentration of *p*-chloromercuribenzoate (2×10^{-5} M) which completely inhibits the *E. coli* enzyme activity (5) enhances the activity of the *B. subtilis* enzyme substantially. Iodoacetate, another sulfhydryl inhibitor, has a similar activating effect. In contrast, 2-mercaptoethanol which permits the *E. coli* reaction to go to completion (5) is inhibitory to the *B. subtilis* DAHP synthetase activity at

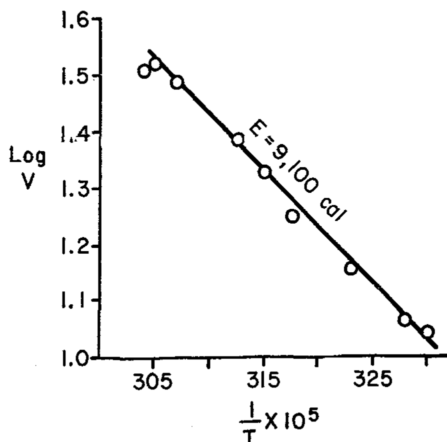

Fig. 4. Arrhenius plot. The data (taken from Fig. 3, 11.1-fold purified preparation) include initial velocity determinations at temperatures between 30 and 56°, expressed as ΔA_{549} per min plotted against reciprocal of temperature (Kelvin scale). DAHP synthetase was not inactivated during the 5-min incubation period below temperatures of 55°. *E* equals activation energy.

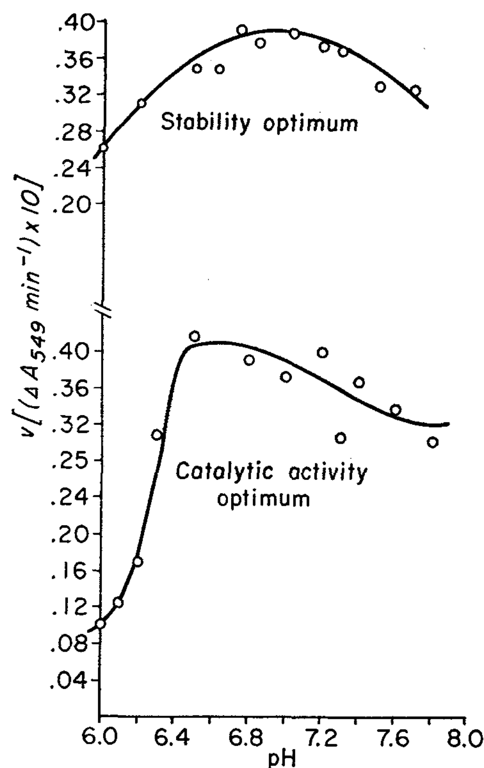

Fig. 5. Variation of reaction velocity (expressed as ΔA_{549} per 10 min) with hydrogen ion concentration. The optimal pH for enzyme stability was determined by storage of the enzyme preparation at 4° for 18 hours at the indicated pH followed by a 20-fold dilution to pH 6.5 before the assay of activity by the usual procedure. The optimal pH for catalytic activity was determined by diluting the extract 20-fold in 0.04 M phosphate buffer in 0.10 M KCl to give the pH values indicated on the *abscissa* and assaying for activity by the usual procedure. Each *point* represents an average determination from two experiments. Extract, 500 μg per ml of protein; a 50 to 55% $(NH_4)_2SO_4$ salt fraction prepared according to Purification Procedure 3.

concentrations above 10^{-5} M. These effects of thiol reagents upon catalytic activity also influence the sensitivity of the enzyme to the feedback inhibitors, chorismate and prephenate; a detailed account of this phenomenon is given in the accompanying paper (11). The latter effects, as well as the effect of other compounds upon enzyme activity are summarized in Table II. DAHP synthetase requires no metal ion cofactors and 10^{-4} M EDTA does not affect activity. EDTA was used in the purification procedure to protect the partially purified enzyme (Fraction V). Urea at 0.8 M inactivates the enzyme as shown in Table II. Comparisons of the addition of urea to numerous preparations at various stages of purification always resulted in similar inactivation curves. Chorismate and prephenate inhibit DAHP synthetase to a maximum of about 80% at 37°. The specificity, relationship to metabolic control, and kinetic analysis of these inhibitions has been published elsewhere (3, 4, 11). The evaluation of the affinity constant, K_m, for the substrates by the use of the double reciprocal plot is shown in Fig. 6. The K_m for erythrose-4-P is 1.1×10^{-3} M and the K_m for P-enolpyruvate is 4.8×10^{-4} M.

Molecular Weight Determinations—The molecular weight of DAHP synthetase was determined by gel filtration according to the method of Andrews (8) with the use of Sephadex G-100 and G-200. The elution profile shown in Fig. 7 takes the form of a

TABLE II

Modifiers of DAHP synthetase activity

Crude, 11.5-fold, and 51-fold refer to degree of purification of extracts prepared from strain SB 167. The crude extract had a specific activity of 135 units per mg. Procedures 1 and 3 were used for purification (11.5-fold and 51-fold, respectively). Additions were made as solutions with pH adjusted to 6.5 to 6.8. All compounds were incubated with the enzyme at 37° for 10 min (except urea, 20 min) before the addition of substrates. The three enzyme preparations were diluted appropriately so that control reaction velocities were comparable. Reaction time was 10 min at 37°.

Addition	Concentration	Inhibition or inactivation			Activation		
		Crude	11.5-fold	51-fold	Crude	11.5-fold	51-fold
	M	%	%	%	%	%	%
EDTA	1.0×10^{-4}	2	4				
2-Mercapto-ethanol[a]	5.0×10^{-3}	55		49			
Urea	8.0×10^{-1}	31	39	41			
Chorismate[b]	1.0×10^{-3}	66	77	71			
Prephenate[b]	2.0×10^{-4}	79	75	75			
P-Chloromer-curibenzoate	2.0×10^{-5}				103		108
Iodoacetate	2.0×10^{-5}				108		100

[a] Corrected for inhibition of assay system.
[b] Isolation of these metabolites previously described (3).

Evidence for One DAHP Synthetase

Inhibition Analysis—The DAHP synthetase in crude extracts of *B. subtilis* conceivably could contain a mixture of two isoenzymes, one inhibited by chorismate and the other by prephenate. The fact that inhibition on the order of 80% can be caused by either effector could be explained by a nonspecific inhibition sometimes observed at high effector concentrations (1). However, fractionation procedures resulting in 77-fold purification compared to the activity of crude extracts have never been observed to abolish or to alter the relative sensitivity of the enzyme to these inhibitors (see Table II). In this context it is also observed that strains of *B. subtilis* differing in the relative sensitivity of DAHP synthetase to one inhibitor show a corresponding difference in sensitivity to the other inhibitor (11).

Homogeneity of Properties—There have been no indications of disparities or irregularities in the kinetic parameters of reaction velocity under conditions that might be expected to reveal a heterogeneous catalyst (isoenzymes). For example, substrate saturation curves are smooth and do not reflect the discontinuity expected of enzymes with dissimilar K_m constants. Smooth curves with single maxima rather than bimodal curves characterize the relationships of reaction velocity to temperatures (Figs. 2, 3, and 4) or pH (Fig. 5).

Fractionation Studies—Although the enzyme activity is broadly distributed through ammonium sulfate salt fractions taken between 45 to 75% of saturation at 0°, there is no difference in the sensitivities of these enzyme fractions to inhibition. Fur-

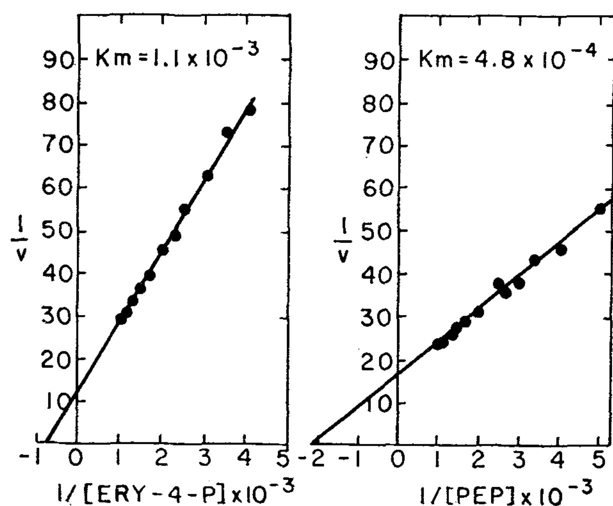

FIG. 6. Double reciprocal plot of erythrose-4-P (*left*) and P-enolpyruvate (*right*) concentrations and initial reaction velocities. P-enolpyruvate concentration (*left*), 2.0 mM. Erythrose-4-P concentration (*right*), 2.0 mM. The velocity (v) is expressed as micromoles of DAHP per min per ml. K_m values are indicated on the figure.

single smooth symmetrical peak, and DAHP synthetase and serum albumin dimer are eluted as coincident peaks. The elution volume corresponding to DAHP synthetase activity led to the calculation of 134,000 as its molecular weight. This result compares favorably with a molecular weight of 129,000 determined by the sucrose density gradient technique of Martin and Ames (9).

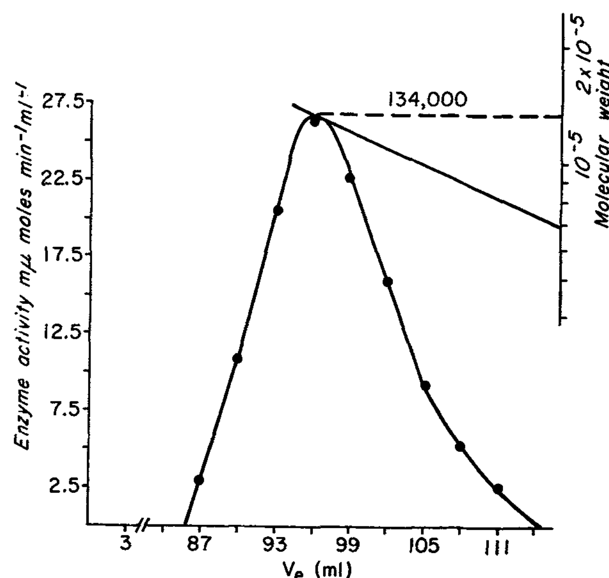

FIG. 7. Determination of DAHP synthetase molecular weight on Sephadex G-100. Plot of elution volume, V_e, against enzyme activity. *Right ordinate* shows molecular weight estimation based on the calibration of the column with cytochrome *c*, serum albumin, and serum albumin dimer. The *line* passing through the *peak* of DAHP synthetase activity and intersecting the *right ordinate* represents the line passing through the reference standard proteins in a plot of V_e against log molecular weight. The interpolated estimation of DAHP synthetase molecular weight is 134,000. The column was equilibrated and eluted with 0.05 M phosphate buffer, pH 7.5, in 0.10 M KCl. Extract from strain SB 167, treated as through Step III of Table I, containing 1.1 mg of protein and a specific activity of 396 mμmoles per min per mg was added to the column in a volume of 1.0 ml.

thermore, subsequent fractionation steps such as gel filtration or DEAE-cellulose chromatography yielded identical activity profiles regardless of which ammonium sulfate fraction was used. It is also clear that there is no detectable association of enzyme activity with proteins of different molecular weights. The elution of the single peak of activity seen in Fig. 7 represents a substantial portion of the activity in the crude extract. (About 90% of the activity applied to the column was recovered in the eluates.)

As previously mentioned, the use of DEAE-cellulose represented the most effective purification procedure and Fig. 8 shows the elution profile of DAHP synthetase activity from this resin. Again the activity is eluted as a single peak in a linear gradient of KCl in potassium phosphate buffer, pH 6.8. A similar profile was obtained with the use of a linear gradient of KCl with either DEAE-cellulose or TEAE-cellulose resins. However, there was a consistent separation of DAHP synthetase in the peak region in each of several chromatographic runs (about tube 80, Fig. 8), and this could be exaggerated by the use of extremely shallow salt gradients (between 0.10 M KCl and 0.30 M KCl). Enzyme taken from eluates corresponding to the shoulder regions of these peaks did not reveal any differences in properties such as sensitivity to inhibitors, urea inactivation, storage stability, or pH optimum. It is not known whether this effect is a consequence of a repeatably reproduced artifact from the column or whether it reflects some unappreciated heterogeneity of the enzyme molecule that is not obviously related to its control by feedback inhibition. Because of the relative lability of the enzyme after this purification step, enzyme activity in pooled bordering fractions was not recovered successfully in eluates from a second passage through the column.

Genetic Evidence—One line of evidence favoring a single enzyme form of DAHP synthetase was the existence of a number of independently isolated mutants which are blocked in the DAHP synthetase reaction. To our knowledge no mutants which are deficient in this enzyme activity are know to exist in *E. coli*. Presumably, the isoenzymes would be independently mutable and, therefore, mutants lacking DAHP synthetase activity could only result from three independent mutations, an extremely unlikely event. Two of the *B. subtilis* mutants, strains SB 155 and WB 1041, are nonleaky enzyme blocks. The third, strain SB 163, is a leaky mutant. All of these mutations revert readily to wild type. The leaky strain, SB 163, produces barely detectable levels of enzyme in extracts (about 5% of the residual nonrepressible activity of wild type strains). If *B. subtilis* had isoenzyme DAHP synthetases, then mutants deficient for one of them would probably be slow growing strains because of the residual activity of the remaining isoenzyme. Since SB 163 fits this description, the nature of its leakiness was investigated further. When this mutant is grown on unsupplemented minimal medium, small colonies of two distinct sizes, designated S-8 and S-10, appear with high frequency (about 10^{-5}). Both of these revertant types grow more slowly than wild type, and they may be suppressor revertants. The DAHP synthetase activity *in vitro* of both SB 163 (S-10) and SB 163 (S-8), slow and faster growing revertant types, respectively, is no greater than that of extracts prepared from the parental mutant, SB 163. Data summarized in Table III suggest that the reversion involves enhanced enzyme stability. When the enzyme assay is done with toluenized whole cells rather than with dialyzed extracts, the

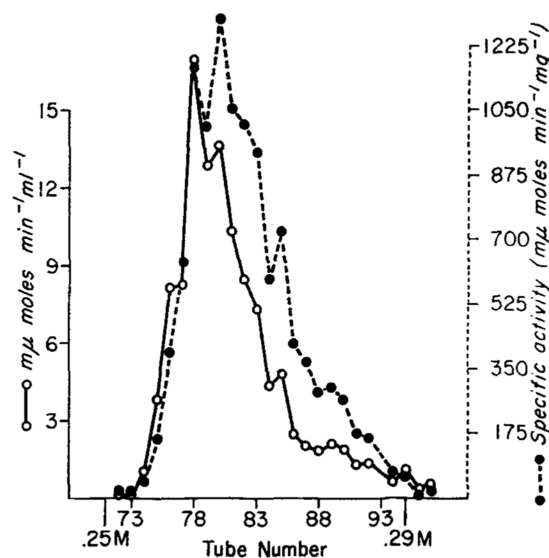

FIG. 8. DEAE-cellulose elution profile. Extract from strain WB 698b, treated as through Step III of Table I, containing 1.0 mg of protein and a specific activity of 174 mμmoles per min per mg in a volume of 1.0 ml. The column dimensions were 2 × 26 cm; flow rate, 12.5 ml per hour; buffer, 0.05 M phosphate, pH 6.8; gradient, 0.05 M KCl (0.04 M phosphate, pH 6.8) to 0.50 M KCl (0.04 M phosphate, pH 6.8); eluate fractions, 1.0 ml; temperature, 4°. DAHP synthetase was eluted as the trailing edge of the second of two major peaks of protein. Each experimental point is an average of two DAHP synthetase assays. Recovery of the DAHP synthetase activity applied to the column was approximately 95%.

TABLE III

Unstable enzyme activities of mutant strains

Cultures (10 ml) were grown with shaking at 37° in a minimal salts medium to middle logarithmic phase growth and treated as described in "Materials and Methods" for whole cell assays. SB 163 is a shikimic acid-requiring auxotroph which is deficient in DAHP synthetase activity.

Strain	Extract specific activity	Toluenized cells specific activity	Generation time
	mμmoles/min/mg	*mμmoles/min/mg*	*min*
SB 163	0.3	0.8	475
SB 163(S-10)	0.3	3.4	210
SB 163(S-8)	0.3	4.8	100
SB 491[a]	5.4	5.7	80

[a] Wild type prototroph.

enzyme activities of the leaky mutant and its two revertant derivatives vary in rough proportion to the growth rates of these strains in minimal medium. Thus, the original mutant and its two revertant derivatives possess growth rates in minimal medium which are limited by their DAHP synthetase activity as indicated by the correlation between growth rate and enzyme activity (Table III). The nearly complete loss of activity in extracts of all three mutant strains indicates that the primary mutation is one which results in an unstable enzyme rather than loss of an isoenyzme activity. The two revertant classes apparently possess slightly more stable enzymes. The enzyme from these strains has not yet been studied, but it is possible that

such studies could provide additional information in order to stabilize the enzyme *in vitro*. We have carried out experiments to show that the instability is not a matter of temperature sensitivity, that the strain does not make an inhibitor substance, and that the enzyme activity observed in whole cells cannot be duplicated in extracts by the addition of boiled extract from wild type. The other revertible mutants, strains SB 155 and WB 1041, have no detectable DAHP synthetase activity in extracts or in toluenized cells. There can be no doubt that a single genetic event abolishes all detectable enzyme activity in these mutant strains.

DISCUSSION

Physical differences between isoenzyme proteins[3] leading to the binding of different allosteric effectors often are accompanied by other variations in properties which can be used to separate the isoenzymes. To date, well studied systems of isoenzyme-mediated regulation (1, 12, 14–17) consist of isoenzymes which are easily separated, even by rather crude fractionation procedures. In addition, such catalytically similar proteins usually differ in properties such as sensitivity to inactivation by heat. Nevertheless, not many examples have been studied and it must be conceded that probability favors the detection of systems of highly individual isoenzymes. Therefore, in order to examine the possible occurrence of isoenzymes more thoroughly, we have presented fractionation data which would have detected relatively subtle differences in enzyme properties. Invariably, the examination of reaction velocity as a function of pH and temperature or the use of inactivating agents (such as heat treatment or urea) yielded results entirely consistent with expectations for a single homogeneous catalytic species of enzyme. The latter was true whether crude or partially purified preparations were used. In the course of purification procedures with ammonium sulfate, gel filtration, and DEAE-cellulose there was never any separation of discrete enzyme activities differing in their relative sensitivity to feedback effectors or in any of the other various properties which were measured. The interpretation of these results favoring homogeneity of the enzyme with respect to control by feedback inhibition is further strengthened because the stability of the enzyme permitted various fractionation procedures to be carried out with relatively little loss of total activity (Figs. 7 and 8). The relative sensitivity of enzyme activity to prephenate and chorismate was invariant in numerous preparations from several strains throughout a wide range of purification.

We have described (3, 4) a new pattern of allosteric control which is consistent with effective regulation of the biosynthetic pathway by the inhibition of a single DAHP synthetase. Hence, the explication of the control system *per se* represents a logical basis for a single enzyme. The third line of evidence for a single DAHP synthetase is genetic. In *E. coli* it has been possible to obtain isoenzyme mutants only through the application of special nutritional procedures (18). The acquisition of single step *B. subtilis* mutations that are deficient for DAHP synthetase activity is most consistent with expectations for a single species of enzyme.

In concert, these several lines of evidence strongly suggest that the DAHP synthetase of *B. subtilis* is a single enzyme.[4]

[3] We refer here to enzyme multiplicity known to be the mechanism for the control by feedback inhibition in divergent metabolic pathways (12) rather than to the more general "isoenzymes" decribed by Markert and Møller (13).

[4] Although we have shown that there is no isoenzyme system of DAHP synthetases in *B. subtilis* as there is in *E. coli*, this by no means suggests that isoenzymes may not be found wherever they are appropriate in other regulatory systems of *B. subtilis*. For example, pyrroline dehydrogenase isoenzymes, subject to regulation by proline and arginine, have been described in *B. subtilis* (17).

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