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3-DEOXY-D-*arabino*-HEPTULOSONATE-7-PHOSPHATE SYNTHASE OF *STREPTOMYCES AUREOFACIENS* Tü 24

I. PARTIAL PURIFICATION AND PROPERTIES

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SUMMARY

1. 3-Deoxy-D-*arabino*-heptulosonate-7-phosphate synthase (7-phospho-2-oxo-3-deoxy-D-*arabino*-heptonate D-erythrose-4-phosphate-lyase (pyruvate phosphorylating), EC 4.1.2.15, DAHP synthase), the first enzyme of the aromatic amino acid pathway, has been purified 170 fold from *Streptomyces aureofaciens* Tü 24. DAHP synthase is a single enzyme with a molecular weight of 97 000.

2. The enzyme has a broad pH stability optimum between pH 6 and 8. The catalytic activity has a pH optimum of 7. EDTA causes a partial inactivation of the enzyme, which can be reversed by Co^{2+}. The activation energy of the enzymatic reaction was estimated to be 16 500 cal/mole. Heat inactivation occurs above 50°. Phosphoenolpyruvate protects the enzyme against heat inactivation, whereas the presence of erythrose 4-phosphate increases the rate of inactivation.

3. The initial velocity data follow regular Michaelis–Menten kinetics without any detectable kinetic evidence for subunit interaction. On the basis of kinetic experiments the mechanism of enzyme action is ping-pong, and the first substrate is phosphoenolpyruvate. The absolute Michaelis constants of the enzyme have been determined to be $3 \cdot 10^{-4}$ M for phosphoenolpyruvate and $1.6 \cdot 10^{-4}$ M for erythrose 4-phosphate.

4. Quantitative determination of phosphoenolpyruvate and erythrose 4-phosphate by the use of a DAHP synthase preparation of *Streptomyces aureofaciens* is described. The method shows a deviation of $\pm 0.7\%$ from the theoretical values.

INTRODUCTION

3-Deoxy-D-*arabino*-heptulosonate-7-phosphate synthase (7-phospho-2-oxo-3-deoxy-D-*arabino*-heptonate D-erythrose-4-phosphate-lyase (pyruvate phosphorylating), EC 4.1.2.15, DAHP synthase) catalyzes the first reaction in the biosynthesis

Abbreviations: DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; erythrose-4-*P*, erythrose 4-phosphate.

of aromatic amino acids and some other aromatic compounds in microorganisms and plants. This first step in the aromatic pathway is the formation of DAHP and P_i from phosphoenolpuryvate and erythrose-4-*P*. Enzymological comparisons of control mechanisms in a variety of organisms have revealed the remarkable versatility which characterizes the control of activity of enzyme systems catalyzing identical sequences of biochemical reactions. The multibranched aromatic and biosynthetic pathway in *Escherichia coli* K 12 is controlled by specific interactions of tyrosine, phenylalanine or tryptophan with three cognate isoenzymes, as found by DOY AND BROWN[1], BROWN AND MAAS[2] and WALLACE AND PITTARD[3,4]. As shown by LINGENS *et al.*[5] two isoenzymes are present in *Saccharomyces cerevisae*, sensitive to phenylalanine and tyrosine, whereas in *Bacillus subtilis* the DAHP synthase is a single protein, sensitive to chorismate and prephenate, as found by JENSEN AND NESTER[6].

The present paper describes the partial purification and some properties of the DAHP synthase of *Streptomyces aureofaciens* Tü 24, which is a single protein, sensitive only to L-tryptophan.

MATERIALS AND METHODS

Chemicals

The inorganic chemicals used were of analytical reagent grade. Glycerol, L-tryptophan and 2-thiobarbituric acid were obtained from Merck, Darmstadt, dithiothreitol (Cleland's reagent), chymotrypsinogen (bovine pancreas), albumin (bovine plasma) and γ-globulin (bovine) from Calbiochem, Los Angeles, Calif. *p*-Hydroxymercuribenzoate was obtained from Carl Roth, Karlsruhe, Sephadex G-100 and G-200 from Pharmacia, Uppsala, and DEAE-cellulose DE 52 Whatman from Hormuth–Vetter, Wiesloch, alumina powder (Alcoa 305) bacteriological grade was obtained from Alcoa Deutschland, Frankfurt. Phosphoenolpyruvate was prepared according to CLARK AND KIRBY[7] and erythrose-4-*P* according to BALLOU[8].

DAHP was a gift from Prof. Sprinson, New York. D-Erythrose 4-phosphate diethylacetal, barium salt, was a gift from Boehringer, Mannheim.

Buffers

Buffer A: 0.1 M potassium phosphate (pH 7.0)–10^{-5} M dithiothreitol.

Buffer B: 0.01 M potassium phosphate (pH 7.0)–10^{-5} M dithiothreitol, 0.15 M KCl–$2.5 \cdot 10^{-4}$ M $CoCl_2 \cdot 6\ H_2O$.

Buffer C: 0.05 M potassium phosphate (pH 7.0)–10^{-5} M dithiothreitol–0.05 M $(NH_4)_2SO_4$–$2.5 \cdot 10^{-5}$ M $CoCl_2 \cdot 6\ H_2O$.

Buffer D: 0.05 M potassium phosphate (pH 7.0)–10^{-5} M dithiothreitol–0.05 M $(NH_4)_2SO_4$–$2.5 \cdot 10^{-4}$ M $CoCl_2 \cdot 6\ H_2O$.

In the presence of $2 \cdot 10^{-4}$ M L-tryptophan, DAHP synthase activity could not be determined in Tris–HCl buffer, because of the interference of tryptophan with the chemical determination of DAHP. This was circumvented by the use of phosphate buffer. In addition the enzyme activity was far less stable in Tris–HCl buffer. Preliminary experiments showed no differences in kinetic patterns determined in phosphate buffer or Tris–HCl buffer. Therefore phosphate buffers were used throughout. Phosphate buffer C containing $(NH_4)_2SO_4$ yields a better recovery of enzyme activity by chromatography on Sephadex G-100 or G-200 than phosphate buffer containing

KCl, which was used by ANDREWS[12]. For this reason $(NH_4)_2SO_4$ was also added in Buffer D.

Organism and growth conditions

S. aureofaciens Tü 24 was a gift from Prof. H. Zähner, Tübingen. The organism was grown with vigorous stirring and an air flow of 10 l/min in a 10-l fermentor at 29°. The minimal medium contained 10 g glycerol, 1 g NH_4Cl, 1 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7 H_2O$, 0.5 g NaCl and 1 g $CaCO_3$ per l. After 45 h the cells were harvested at the exponential growth phase by centrifugation, washed once with Buffer A and stored at −25°.

DAHP synthase assay

The activity of DAHP synthase was assayed by the chemical determination of DAHP described by SPRINSON *et al.*[9]. This method was used with slight modifications.

The standard reaction mixture contained 0.425 μmole phosphoenolpyruvate, 0.3 μmole erythrose-4-*P* and protein in a total volume of 1 ml of Buffer D. After incubation at 30° for 10 min the reaction was stopped by the addition of 100 μl of 50% (w/v) trichloroacetic acid and the precipitated protein was pelleted by centrifugation. An aliquot of the supernatant (500 μl) was removed and treated with 250 μl of 25 mM HIO_4 in 62.5 mM H_2SO_4. After incubation at 37° for 30 min according to DOY AND BROWN[1] 500 μl of 2% sodium arsenite in 0.5 M HCl were added, followed by 2 ml of 0.3% thiobarbituric acid solution when the yellow colour had disappeared. The test tubes were capped with marbles and placed in a boiling waterbath for 10 min; they were then kept in a waterbath at 55° before measuring the absorbance at 549 nm.

The amount of DAHP was calculated by using a value of $6.8 \cdot 10^4$ for the molar absorbance coefficient of DAHP at 549 nm, which had been determined with an authentic sample of DAHP under the conditions described above.

The initial velocity, *v*, is defined as nmoles DAHP formed per min per ml of the reaction mixture. One unit of enzyme represents the formation of 1 μmole DAHP per min per ml of the reaction mixture under standard conditions.

Specific activity is expressed as milliunits per mg of protein.

A typical family of activity curves obtained at different concentrations of substrates as a function of enzyme concentration is shown in Fig. 2. Fig. 3 shows the reaction rate as a function of time.

Quantitative determination of phosphoenolpyruvate and erythrose-4-P

Determination of phosphoenolpyruvate. The reaction mixture contained 0.025–0.05 μmole phosphoenolpyruvate, 0.3 μmole erythrose-4-*P* and 0.3–0.5 mg protein of the acetone fractionation step of purification procedure A in a total volume of 1 ml of Buffer D.

Determination of erythrose-4-P. 0.025–0.04 μmole erythrose-4-*P*, 0.425 μmole phosphoenolpyruvate and 0.3–0.5 mg protein of the acetone fractionation step in a total volume of 1 ml of Buffer D.

The reaction mixtures were incubated at 30° for 30 min.

Protein determination

Protein was determined by the method of GROVES *et al.*[10], using the isoabsorbance wavelengths 224 nm and 235 nm, which have been determined for a nucleic acid preparation of *S. aureofaciens*. In crude extracts and $(NH_4)_2SO_4$ fractions this method gave comparable results to that of LOWRY *et al.*[11].

Molecular weight determination with Sephadex G-100

A Sephadex G-100 column (2.5 cm × 40 cm) was prepared according to ANDREWS[12] and equilibrated with Buffer C at 3°. The sample solution of 1 ml was carefully layered under the buffer solution above the gel and 3.4-ml fractions were collected. The flow rate was maintained at 25 ml/h.

RESULTS AND DISCUSSION

Partial purification of the enzyme (A)

All fractionation procedures were carried out at 4°.

Crude extract. Cell-free extracts were prepared by grinding 9 g wet frozen cells mixed with the same weight of alumina powder (Alcoa 305) in a mortar. The suspension was taken up in a double volume of buffer A. Cell debris was removed by centrifugation at 20 000 × *g* for 20 min.

Fractionation with $(NH_4)_2SO_4$. Crude extract was diluted with Buffer A to a final concentration of 10 mg protein per ml. Solid $(NH_4)_2SO_4$ was added with stirring to a final saturation of 30% (ref. 13). The precipitate was removed by centrifugation and solid $(NH_4)_2SO_4$ was added to a final saturation of 50%. The latter fraction contained most of the enzyme activity and was resuspended in Buffer A.

Fractionation with acetone. The $(NH_4)_2SO_4$ fraction was diluted with Buffer A to a final concentration of 20 mg protein per ml. 0.95 ml of acetone per ml of protein

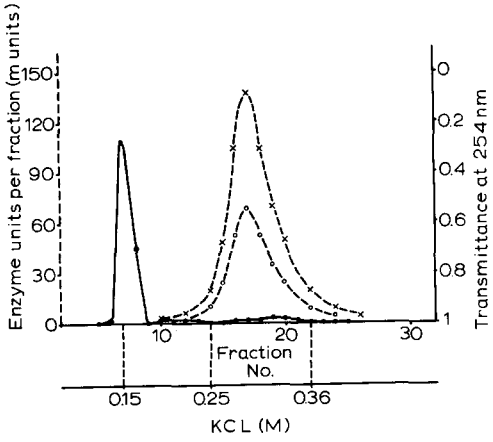


Fig. 1. DEAE-cellulose elution profile. 4 ml of the acetone fraction of procedure A containing 4–7 mg of protein were applied to a column of dimensions 1.5 cm × 30 cm. The flow rate was maintained at 20 ml/h. DAHP synthase was eluted with a linear KCl gradient in Buffer B; fractions of 5.4 ml were collected. Recovery of DAHP synthase activity applied to the column was approximately 90%. × - - - ×, activity of DAHP synthase; ○ - - - ○, activity of DAHP synthase in the presence of $2 \cdot 10^{-4}$ M tryptophan; ●—●, protein.

solution was added with stirring. The precipitate was removed by centrifugation and 0.3 ml of acetone per ml of the original protein solution was added to the supernatant. The precipitated enzyme was removed by centrifugation and dissolved in 4 to 5 ml of Buffer B.

DEAE-cellulose chromatography. 4 ml of acetone fraction with 4 to 7 mg protein were applied to the top of a DEAE-cellulose column (1.5 cm × 30 cm) which had been equilibrated with Buffer B. The enzyme was eluted with a linear salt gradient of 0.15–0.7 M KCl (100 ml Buffer B/100 ml Buffer B + 0.55 M KCl). Fractions of 5.4 ml were collected at a flow rate of 20 ml/h.The enzyme was eluted in a symmetrical peak at a KCl concentration of 0.25–0.36 M as shown in Fig. 1.

A typical purification is summarized in Table I.

TABLE I

ENZYME PURIFICATION

Step	*Fraction*	*Vol. (ml)*	*Total units (m/units)*	*Total protein (mg)*	*Specific activity*	*Yield (%)*
1	Crude extract*	50	3860	495	7.8	100
2	$(NH_4)_2SO_4$ fractionation	9	3840	188	20.5	99
3	Acetone fractionation	4.5	1670	76	220	43
4	DEAE-cellulose	19	470	0.36	1310	12

*From 9 g wet cells.

Another purification procedure (B)

The fractionation procedure outlined in Table I results in a 170-fold purification with a total yield of enzyme activity of 12%. A simpler procedure results in a 14-fold purification with a total yield of 80%. The $(NH_4)_2SO_4$ precipitate between 30 and 50% saturation in the procedure A was redissolved in Buffer C to a final concentration of 20 mg of protein per ml. 4 ml of the enzyme solution were applied to the top of a Sephadex G-200 column (2.5 cm × 60 cm) which had been equilibrated with Buffer C. The enzyme was eluted with Buffer C in a symmetrical peak. Fractions of 7 ml were collected at a flow rate of 25 ml/h.

Both preparations of DAHP synthase show the same Michaelis constants for the substrates phosphoenolpyruvate and erythrose-4-*P*. In practice, enzyme preparations of the simpler purification procedure B were used in most of the experiments described in this paper.

The activity of DAHP synthase in crude extracts of *S. aureofaciens* is inhibited by $2 \cdot 10^{-4}$ M L-tryptophan to about 60% at pH 7 and about 90% at pH 6.3. A detailed account of this phenomenon is given in the accompanying paper. L-Phenylalanine and L-tyrosine do not influence the enzyme activity.

Although the enzyme activity is distributed throughout $(NH_4)_2SO_4$ fractions taken between 30 and 50% saturation, there is no difference in the sensitivity of these enzyme fractions with regard to inhibition. During fractionation procedures resulting in a 170-fold purification, compared to the activity of the crude extract, the

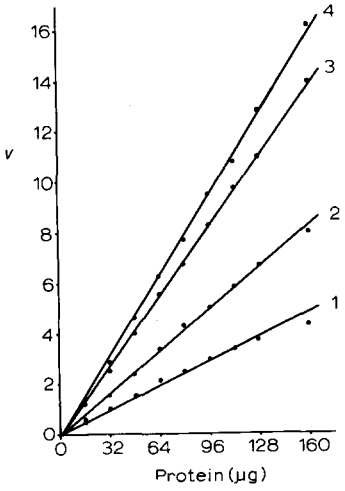
TABLE II

EFFECT OF Co^{2+} ON THE ENZYME ACTIVITY

An enzyme preparation purified according to the procedure B was used. 60 μg protein, 0.3 μmole erythrose-4-*P* and additions at the concentrations indicated in a total volume of 1 ml of 0.05 M potassium phosphate buffer (pH 7.0) + 0.05 M $(NH_4)_2SO_4$ were incubated at 30° for 5 min or 15 min*. The enzymatic reaction was started by the addition of 0.425 μmole phosphoenolpyruvate in 50 μl 0.05 M potassium phosphate buffer, pH 7.0.

Addition		*Relative enzymic activity*
EDTA (M)	*Co^{2+} (M)*	
—	—	1
10^{-4}	—	0.67
10^{-3}	—	0.58
—	$2.4 \cdot 10^{-4}$	1.13
10^{-4}	$2.4 \cdot 10^{-4}$	1.15
10^{-3}	$2.4 \cdot 10^{-4}$	0.6
—	$1.2 \cdot 10^{-3}$	1.13
10^{-4}	$1.2 \cdot 10^{-3}$	1.17
10^{-3}	$1.2 \cdot 10^{-3}$	1
*Dithiothreitol	10^{-5}	1.3

extent of inhibition by tryptophan remained unchanged. Fractionation on DEAE-cellulose and by gel filtration also yielded symmetrical peaks of enzyme activity. Therefore we conclude that DAHP synthase of *S. aureofaciens* is a single enzyme.



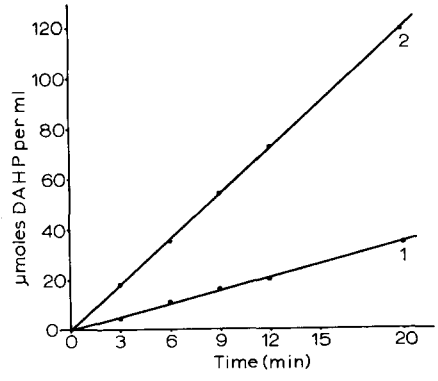


Fig. 2. Variation of enzyme activity as a function of enzyme concentration at various substrate concentrations. An enzyme extract purified according to the procedure B was used. The reaction volume was 1 ml of Buffer D. (1) 0.085 μmole phosphoenolpyruvate and 0.06 μmole erythrose-4-*P*; (2) 0.17 μmole phosphoenolpyruvate and 0.12 μmole erythrose-4-*P*; (3) 0.425 μmole phosphoenolpyruvate and 0.3 μmole erythrose-4-*P*; (4) 0.85 μmole phosphoenolpyruvate and 0.6 μmole erythrose-4-*P*.

Fig. 3. Plot of DAHP formed as a function of time at 30°. 150 μg protein purified according to the procedure B in 1 ml of Buffer D. (1) 0.085 μmole phosphoenolpyruvate and 0.06 μmole erythrose-4-*P*; (2) 0.425 μmole phosphoenolpyruvate and 0.3 μmole erythrose-4-*P*.

Factors influencing the enzyme activity

The DAHP synthase of *S. aureofaciens* is slightly stimulated by Co^{2+} after chromatography on Sephadex G-200. The addition of EDTA to the reaction mixture results in a 40% loss of activity, which can be prevented completely by Co^{2+}. Dithiothreitol stabilizes the enzyme during incubation at 30° for 15 min. These results are summarized in Table II. The enzyme is inhibited completely by $2 \cdot 10^{-5}$ M *p*-hydroxymercuribenzoate.

The DAHP synthase of *S. aureofaciens* requires Co^{2+} for full activity and possesses one or more sulfhydryl groups which are essential for binding the substrates or for catalytic function. A third possibility is that the sulfhydryl is important for structural reasons. Therefore all tests were done in the presence of $2.5 \cdot 10^{-4}$ M Co^{2+} and 10^{-5} M dithiothreitol. The enzyme displays uncomplicated reaction kinetics under the conditions of assay described; the reaction rate is a linear function of protein concentration within a limited range of enzyme concentration, as shown in Fig. 2 for various concentrations of substrates. No disproportional loss of activity occurs with the dilution of extracts. Fig. 3 indicates that the linearity of the reaction rate is maintained for at least 20 min at 30°.

The effect of pH on the reaction velocity and enzyme stability

The influence of H^+ concentration upon enzyme activity and enzyme stability

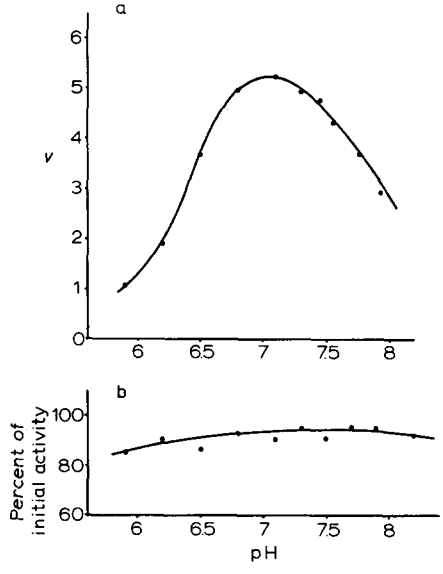


Fig. 4. (a) Variation of reaction velocity with H^+ concentration. An enzyme preparation purified according to the procedure B was used. The reaction mixture contained 60 μg of protein, 0.425 μmole phosphoenolpyruvate and 0.3 μmole erythrose-4-*P* in 1 ml of 0.3 M potassium phosphate buffer at various pH values. (b) The optimal pH for enzyme stability was determined by storage of the enzyme preparation at 30° for 2 h in 0.1 M potassium phosphate buffer + 10^{-5} M dithiothreitol + $2.5 \cdot 10^{-4}$ M Co^{2+} at the indicated pH. Aliquots of 100 μl, containing 90 μg of protein, were removed and the enzymatic activity determined in 0.3 M potassium phosphate buffer (pH 7.0) + 10^{-5} M dithiothreitol + $2.5 \cdot 10^{-4}$ M Co^{2+}. The reaction mixture contained 0.425 μmole phosphoenolpyruvate, 0.3 μmole erythrose-4-*P* and protein in a total volume of 1 ml. The activity of a sample, which had been stored at 0° and pH 7.0, was taken as 100%.

are shown in Fig. 4. DAHP synthase activity increases rapidly with increasing pH to an optimum pH of 7.0. Further increase in pH to 8.0 results in a strong decrease in activity. The enzyme is quite stable at 30° in the pH range 6.0–8.0 for at least 2 h. Therefore the enzyme is stable at H^+ concentrations permitting only low reaction rates, *e.g.* pH 6.0. The optimal pH for maintenance of enzyme stability was 7.0–7.5. For this reason enzyme purification procedures were carried out at pH 7. Enzyme activity was determined at this pH, which is the optimal pH of catalytic activity.

Substrate saturation kinetics

The initial velocity of DAHP synthesis at a fixed concentration of erythrose-4-*P* and various concentrations of phosphoenolpyruvate follows regular Michaelis–Menten kinetics. The dependence of the initial rate of DAHP formation on erythrose-4-*P* concentration in the presence of a fixed concentration of phosphoenolpyruvate also follows regular Michaelis–Menten kinetics, which yield straight lines in Lineweaver–Burk plots. No deviations from linearity are observed and there is no kinetic evidence of cooperative subunit interaction.

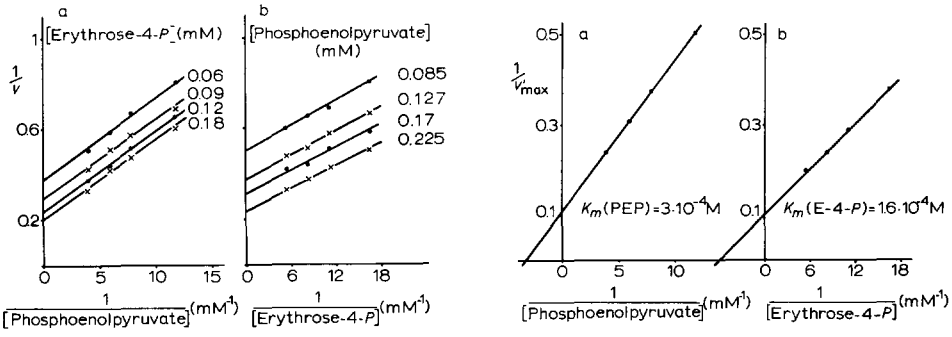


Fig. 5. Double reciprocal plot of the initial rate of DAHP synthesis (a) *vs.* phosphoenolpyruvate concentration in the presence of a series of fixed concentrations of erythrose-4-*P* and (b) *vs.* erythrose-4-*P* concentration in the presence of a series of fixed concentrations of phosphoenolpyruvate. The reaction mixture contained 15 μg protein, purified according to the procedure A, phosphoenolpyruvate and erythrose-4-*P* as indicated in 1 ml of Buffer D.

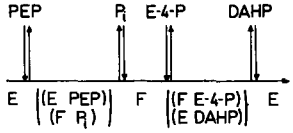
Fig. 6. Secondary plot of the data from Fig. 5 for determination of the absolute Michaelis constants of the enzyme. The graphical analysis yields the absolute Michaelis constants $-1/K_m$(PEP) and $-1/K_m$(E-4-*P*) as intercepts on the abscissa. (a) Determination of $-1/K_m$(PEP) from the data of Fig. 5b. (b) Determination of $-1/K_m$(E-4-*P*) from the data of Fig. 5a.

A set of parallel lines is obtained from double reciprocal plots of the initial rate of DAHP formation against phosphoenolpyruvate concentration for four fixed concentrations of erythrose-4-*P* (Fig. 5a). Double reciprocal plots of the initial velocity of DAHP synthesis against erythrose-4-*P* concentration for four fixed concentrations of phosphoenolpyruvate also give a set of parallel lines (Fig. 5b). The intercepts on the ordinate are the reciprocals of the apparent maximal velocities $1/v'_{max}$. Absolute Michaelis constants of the DAHP synthase for phosphoenolpyruvate and erythrose-4-*P* are determined graphically from secondary plots of these data.

A double reciprocal plot of the apparent maximal velocities against erythrose-

4-*P* concentration from the data of Fig. 5a, gives a straight line with an intercept on the abscissa corresponding to the reciprocal of the absolute Michaelis constant for erythrose-4-*P* (Fig. 6b). The absolute Michaelis constant for phosphoenolpyruvate is also determined from a secondary plot from the data of Fig. 5b, as shown in Fig. 6a. The absolute Michaelis constant of the enzyme for phosphoenolpyruvate, K_m(PEP), is $3 \cdot 10^{-4}$ M and for erythrose-4-*P*, K_m(E-4-*P*), is $1.6 \cdot 10^{-4}$ M.

According to CLELAND[14] a set of parallel lines of $1/v$ *versus* $1/[S]$ plots, when one substrate is varied and the other held constant, indicates a ping-pong mechanism. A ping-pong mechanism means that the enzyme reacts with the first substrate and release of the first product occurs before the addition of the second substrate. During the overall reaction of DAHP synthesis inorganic phosphate is released. It was found by DELEO AND SPRINSON[15] that the P_i released contained the ^{18}O from the C–O–P oxygen of phosphoenolpyruvate and not from $H_2{}^{18}O$. Therefore the first substrate is phosphoenolpyruvate. Phosphoenolpyruvate also stabilizes the enzyme against heat inactivation, whereas erythrose-4-*P* has the opposite effect as shown in Fig. 9. On the basis of the above results, the reaction mechanism may be schematically represented as shown in Scheme 1. E and F are different forms of the enzyme. The



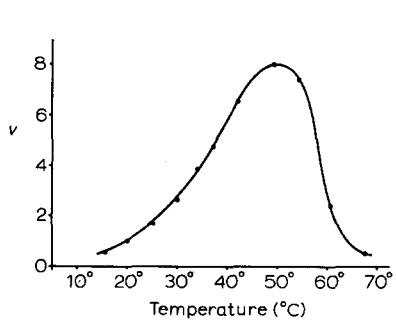
Scheme 1. Graphical representation of the reaction sequence leading to the formation of DAHP. PEP = phosphoenolpyruvate; E-4-*P* = erythrose-4-*P*

first part of the catalytic reaction is the formation of an enzyme–phosphoenolpyruvate complex which forms a pyruvyl–enzyme intermediate F by elimination of P_i.

Similar results were obtained by STAUB AND DÉNES[16,17] with the phenylalanine- and tyrosine-sensitive DAHP synthase in *E. coli* K 12, by NAGANO AND ZALKIN[18] with the tyrosine-sensitive DAHP synthase in *Salmonella typhimurium* and by EBERSPÄCHER *et al.*[19] with the three isoenzymes of DAHP synthase in Claviceps SD 58. The individual steps of the enzymatic reaction are shown as reversible in Scheme 1. According to CLELAND[20], if the step forming the product is reversible, the set of parallel lines should change in the presence of a product to a set of straight lines, intersecting to the left of the vertical axis. The data shown in Fig. 5 are obtained in potassium phosphate buffer, that is in the presence of the product P_i. The set of parallel lines therefore indicates that the formation of P_i and of the enzyme form F from phosphoenolpyruvate and E is an irreversible step. SRINIVASAN AND SPRINSON[21] tried to reverse the overall reaction in the presence of DAHP synthase from *E. coli*, but all attempts were unsuccessful.

Quantitative determination of phosphoenolpyruvate and erythrose-4-P

Phosphoenolpyruvate and erythrose-4-*P* can be determined quantitatively by the use of DAHP synthase of *S. aureofaciens*. Crude extracts and $(NH_4)_2SO_4$ fractions are not useful for this purposes whereas the enzyme preparation of the acetone



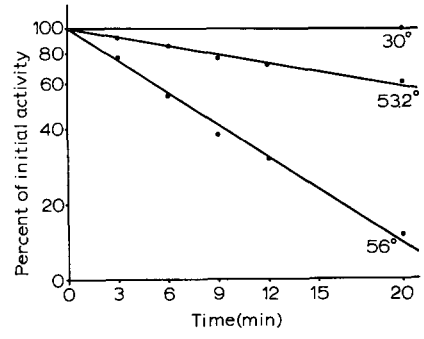
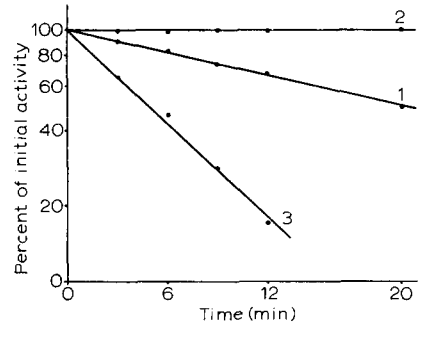


Fig. 7. Dependence of reaction velocity on the reaction temperature. 15 μg of protein, purified according to the procedure A, were used in the standard reaction mixture.

Fig. 8. Heat inactivation of DAHP synthase at various temperatures. The enzyme (820 μg of protein per ml) was incubated in Buffer D at the indicated temperatures. Samples of 100 μl were taken at zero time and at the times indicated. Activity was determined in the standard reaction mixture.

fractionation of the purification procedure A can be used. For the quantitative determination 0.025–0.05 μmole of one substrate is used with an excess of the other. To prove the accuracy of the method standard solutions of phosphoenolpyruvate and erythrose-4-*P* were prepared with phosphoenolpyruvate, potassium salt and erythrose 4-phosphate diethylacetal, barium salt. Erythrose 4-phosphate diethylacetal was hydrolyzed under standard conditions according to the procedure recommended by



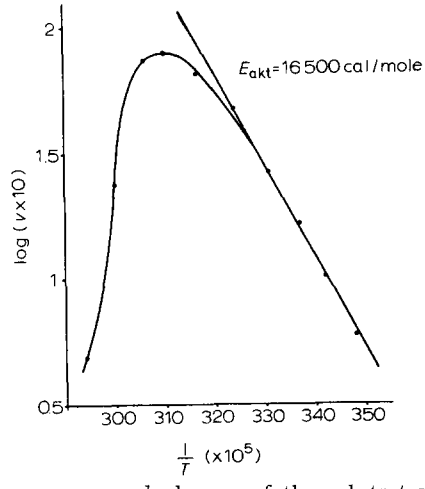


Fig. 9. Heat inactivation of DAHP synthase in the presence and absence of the substrates phosphoenolpyruvate and erythrose-4-*P*. The enzyme (820 μg of protein per ml) was incubated at 55° in Buffer D in the presence and absence of the following concentrations of substrates. (1) Enzyme in the absence of substrates; (2) enzyme in the presence of $4.25 \cdot 10^{-3}$ M phosphoenolpyruvate; (3) enzyme in the presence of $3 \cdot 10^{-3}$ M erythrose-4-*P*. At zero time and the times indicated samples of 100 μl were taken, and the activity was determined in the standard reaction mixture.

Fig. 10. Arrhenius plot of the initial rate ($\log_{10}$) of DAHP formation against reciprocal of absolute temperature. Data are taken from Fig. 7. E_{akt} equals activation energy.

Boehringer. The determination of substrates with DAHP synthase showed deviations of $\pm 0.7\%$ from theoretical values.

Effect of temperature on the activity of the enzyme

The influence of increasing temperature on the initial velocity of the enzymatic reaction is shown in Fig. 7. The temperature optimum of the DAHP synthase activity is 50°. At higher temperatures the enzyme is inactivated with first order kinetics (Fig. 8). Phosphoenolpyruvate confers protection against this inactivation in a concentration of $4.25 \cdot 10^{-3}$ M (Fig. 9). This stabilization of the enzyme by phosphoenolpyruvate is consistent with initial formation of an enzyme–phosphoenolpyruvate complex as shown in Scheme 1. On the other hand, erythrose-4-*P* inactivates the enzyme. This inactivating effect, also observed by NAGANO AND ZALKIN[18] with the tyrosinesensitive DAHP synthase of *S. typhimurium*, seems to be caused by the aldehyde function of erythrose-4-*P*, because erythrose, ribose 5-phosphate as well as ribose also inactivate the enzyme at elevated temperatures but to a lower extent (Tabel III). An inactivation by aldehyde substrates has been reported by LAI *et al.*[22] when studying rabbit muscle aldolase. Results supporting a ping-pong mechanism for the DAHP synthase reaction were found in different microorganisms. In order to obtain other comparable results regarding this bi-substrate reaction the activation energy was determined. Fig. 10 is a standard Arrhenius plot, where the logarithm of the velocity of the enzyme activity is plotted against the reciprocal of the absolute temperature. The activation energy calculated from the negative slope has a value of 16 500 cal/mole. This agrees with the value described for DAPH synthase from *Neurospora crassa* reported by DOY[23] but is considerably higher than that for DAHP synthase from *B. subtilis* reported by JENSEN AND NESTER[6].

TABLE III

INACTIVATION OF DAHP SYNTHASE AT 55° IN THE PRESENCE OF VARIOUS ALDOSES

The enzyme (800 μg of protein per ml) was incubated at 55° in buffer D in the presence of the indicated aldoses at a concentration of $3 \cdot 10^{-3}$ M. At zero time and after 6 min incubation 100-μl aliquots were removed, and the activity was determined in the standard reaction mixture. The activity at zero time was taken as 100%.

Aldose ($3 \cdot 10^{-3}$ *M*)	*Percent of initial activity after incubation*
—	93
Erythrose	68
Erythrose-4-*P*	33
Ribose	80
Ribose-5-*P*	65

Molecular weight determination

The molecular weight of DAHP synthase was determined by gel filtration according to ANDREWS[12] using Sephadex G-100. The column is calibrated with proteins of known molecular weights, chymotrypsinogen (bovine pancreas) mol.wt. 25 000, albumin (bovine plasma) mol.wt. 67 000 and γ-globulin (bovine) mol.wt. 160 000. The DAHP synthase is eluted in a single symmetrical peak (Fig. 11a). From the elution volume corresponding to DAHP synthase activity a molecular

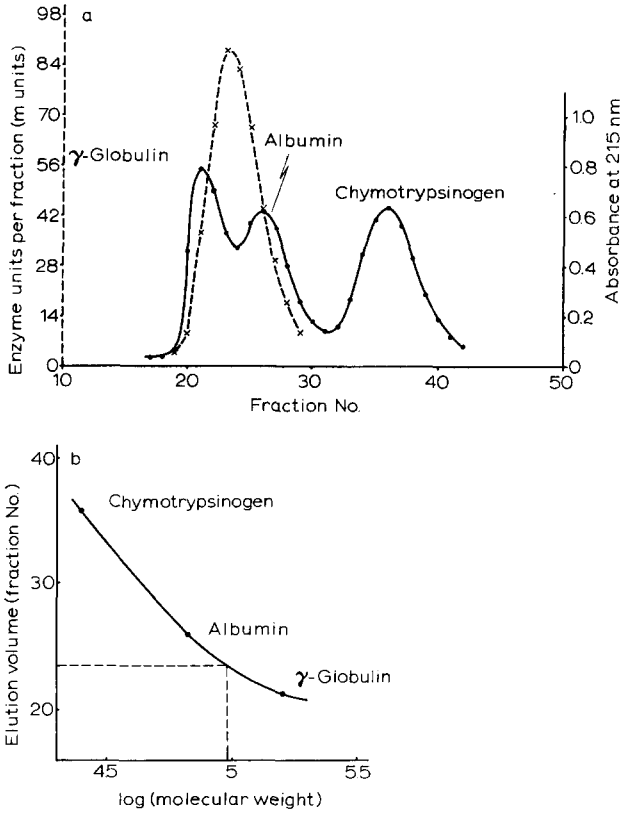


Fig. 11. Determination of the molecular weight of the DAHP synthase on Sephadex G-100. A fraction saturated to 30–50% with $(NH_4)_2SO_4$ (1 ml with 10 mg of protein) was applied to a Sephadex G-100 column (2.5 cm × 40 cm) equilibrated with Buffer C. The enzyme was eluted with Buffer C. The flow rate was maintained at 25 ml/h and fractions of 3.4 ml were collected. (a) Elution profile. (b) Plot of the elution volume against log of molecular weight.

weight of 97 000 was calculated (Fig. 11b). No shift in the elution volume of the enzyme in the presence of either phosphoenolpyruvate or erythrose-4-*P* was detected.

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