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PURIFICATION AND PROPERTIES OF THE 3-DEOXY-D-*ARABINO*-HEPTULOSONATE-7-PHOSPHATE SYNTHASE (PHENYLALANINE SENSITIVE) OF *ESCHERICHIA COLI* K12

II. INHIBITION OF ACTIVITY OF THE ENZYME WITH PHENYLALANINE AND FUNCTIONAL GROUP-SPECIFIC REAGENTS

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SUMMARY

1. The inhibition of activity of the phenylalanine-sensitive 3-deoxy-D-*arabino*-heptulosonate-7-phosphate (DAHP) synthase (7-phospho-2-oxo-3-deoxy-D-*arabino*-heptonate D-erythrose-4-phosphate-lyase (pyruvate-phosphorylating), EC 4.1.2.15) has been studied. The allosteric effector, L-phenylalanine, inhibits the activity of enzyme noncompetitively for both phosphoenolpyruvate (PEP) and erythrose 4-phosphate. Phenylalanine protects the enzyme against the inactivating effect of heat, and the K_i of enzyme for phenylalanine is $3.8 \cdot 10^{-5}$ M.

2. Bromopyruvate, an alkylating structural analog of PEP, irreversibly inactivates the enzyme, and PEP protects the enzyme against the inactivating effect of the alkylating agent. The initial step of the bromopyruvate enzyme interaction obeys the regular saturation kinetics, and the relation between bromopyruvate and PEP is competitive. The K_s of the enzyme for bromopyruvate is 2.0 mM, and the inactivation half-time of the enzyme is 1.5 min.

3. DAHP synthase is a thiol enzyme. *p*-Hydroxymercuribenzoate reversibly, and dithiobis nitrobenzoic acid irreversibly, inactivates the enzyme. PEP protects the enzyme against the inactivating effect of 5,5'-dithio-bis-(nitrobenzoic acid).

4. Metal-binding agents such as cyanide, EDTA, *o*-phenanthroline and 1-nitroso-2-naphthol inhibit the activity of the enzyme. The enzyme contains firmly bound heavy metal, probably Co^{2+}.

Abbrevations: DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; DTNB, 5,5'-dithio-bis-(2-nitrobenzoic acid); erythrose-4-*P*, erythrose 4-phosphate; PHMB, *p*-hydroxymercuribenzoate; PEP, phosphoenolpyruvate.

INTRODUCTION

It was found that the 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthases (7-phospho-2-oxo-3-deoxy-D-*arabino*-heptonate D-erythrose-4-phosphate-lyase (pyruvate-phosphorylating), EC 4.1.2.15) are the allosteric first enzymes of the aromatic amino acid biosynthetic pathways in different organisms[1]. All these enzymes catalyze the same reaction, the formation of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) from phosphoenolpyruvate (PEP) and erythrose 4-phosphate (erythrose-4-*P*)[2]. Although the allosteric effectors or the mode of action of these inhibitors are quite different and characteristic of the different DAHP synthases, some of the catalytic properties of these enzymes seem to be similar. Among others, phenylalanine- and tyrosine-sensitive DAHP synthase of *Escherichia coli*[3,4] and that of *Neurospora crassa*[5] act by a ping-pong mechanism, and the first substrate of the enzyme is PEP. The DAHP synthase of *N. crassa*[5] and the tyrosine-sensitive enzyme of *E. coli*[4] are heavy-metal enzymes containing Co^{2+}. Since the experimental data concerning the structure and mechanism of action of these enzymes are rather incomplete, this paper reports detailed studies on the effect of allosteric inhibitor and other functional group-specific reagents on the activity of the phenylalanine-sensitive DAHP synthase purified from *E. coli* K12.

MATERIALS AND METHODS

Chemicals

The inorganic chemicals used were A.R. grade. PEP barium salt, D-glucose 6-phosphate barium salt, EDTA, L-phenylalanine, and L-tyrosine were purchased from Reanal, Budapest. Thiobarbituric acid, GSH and *p*-hydroxymercuribenzoate (PHMB) were obtained from Fluka, Buchs. β-2-Thienylalanine was obtained from Sigma Chemical Co., St. Louis, Mo., and 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) from EGA Chemie, Steinheim/Albuch. *o*-Phenanthroline hydrate and 1-nitroso-2-naphthol were purchased from Koch-Light Laboratories, Colnbrook. Erythrose-4-*P* was prepared from D-glucose 6-phosphate according to BALLOU[6] and was purified by ion-exchange chromatagraphy, as described previously[7]. Bromopyruvate was prepared chemically[8].

Enzyme preparation

The phenylalanine-sensitive DAHP synthase (isoenzyme 1a) was purified from the sonic extract of *E. coli* K12 as described previously[9]. Protein was determined according to LOWRY *et al.*[10].

Enzyme assay

If not otherwise stated, the standard reaction mixture was used containing 0.5 μmole of PEP, 0.5 μmole of erythrose-4-*P*, 50 μmoles of Tris–HCl buffer (pH 7.5), water and enzyme in a total volume of 1.0 ml. The enzyme assay was carried out at 37°, and the reaction was arrested by the addition of 0.4 ml of 10% trichloroacetic acid. After centrifugation of the precipitated proteins the amount of DAHP formed was determined by the thiobarbituric acid method[2].

RESULTS AND DISCUSSION

Allosteric inhibition of enzyme activity

It was observed earlier that the activity of isoenzyme 1a of DAHP synthase in the extract of *E. coli* K12 is inhibited by phenylalanine[11]. The inhibition of the activity of the purified isoenzyme 1a as a function of phenylalanine concentration is shown in Fig. 1. It was reported earlier that β-2-thienylalanine, a structural analog of phenylalanine, also inhibits the activity of the phenylalanine-sensitive enzyme of *E. coli*[12]. As shown in Fig. 1 the concentration dependence of the inhibitory effect of β-2-thienylalanine is similar to that of phenylalanine. The double reciprocal plots of

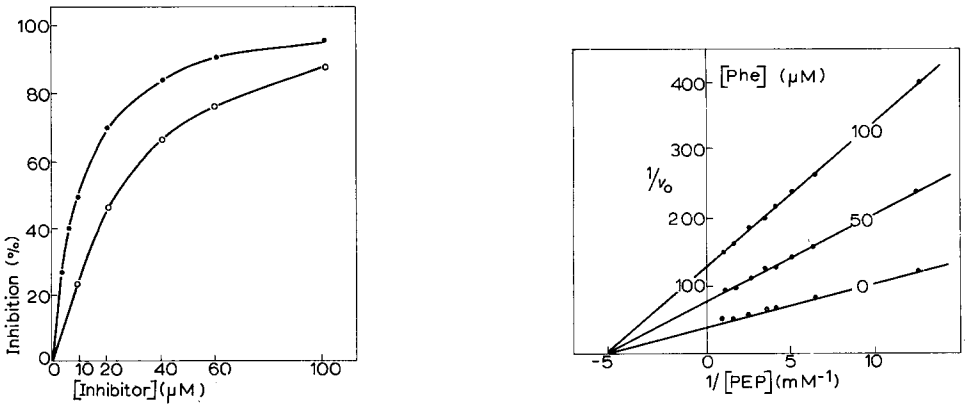
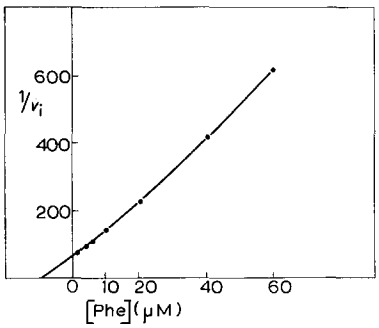


Fig. 1. The effect of phenylalanine and β-2-thienylalanine on the activity of DAHP synthase. The reaction mixtures contain 0.54 μmole of PEP, 0.58 μmole of erythrose-4-*P*, 50 μmoles of potassium phosphate buffer (pH 7.5), 54 μg of protein, and phenylalanine (●—●) or thienylalanine (○—○) as indicated. The reaction mixtures were incubated for 10 min at 37°, and the amount of DAHP formed was determined as described under MATERIALS AND METHODS.

Fig. 2. The effect of phenylalanine on the initial rate of DAHP formation as a function of PEP concentration. The reaction mixtures contained 50 μmoles of potassium phosphate buffer (pH 7.5), 0.40 μmole of erythrose-4-*P*, 30 μg of protein, and PEP and phenylalanine as indicated. Initial velocity, v_0, is expressed as μmoles of DAHP formed per 10 min per ml of reaction mixture.

the initial velocity of DAHP formation as a function of substrate concentration at several inhibitor concentrations are compatible with the regular noncompetitive inhibition for both substrates, as indicated in Figs. 2 and 3. The same type of phenylalanine inhibition was found for the phenylalanine-sensitive DAHP synthase purified from *E. coli* W by SMITH *et al.*[13]. This means conventionally that the enzyme simultaneously binds both the substrates and the inhibitor. In the plot (Dixon plot) of reciprocal of the initial rate of DAHP synthesis as a function of inhibitor concentration there is some deviation from the straight line. A regular noncompetitive inhibition gives a straight line in this case. We also analyzed the data of Fig. 1 according to the Hill equation, as described by MONOD *et al.*[14]. We obtained straight lines for both phenylalanine and thienylalanine, each with a slope of 1.2.

The reason for the irregularity of the inhibition process caused by phenylalanine (which was measured in many cases) may be discussed as a cooperativity developing during the inhibition of the reaction.



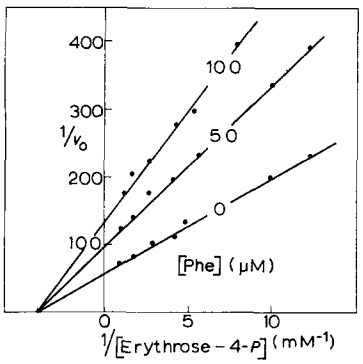


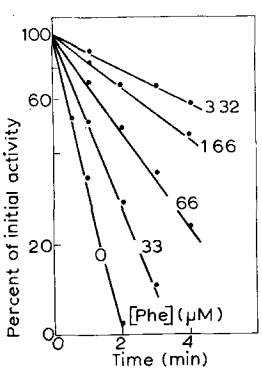
Fig. 3. The effect of phenylalanine on the initial rate of DAHP synthesis as a function of erythrose-4-*P* concentration. The reaction mixture contained 50 μmoles of potassium phosphate buffer (pH 7.5), 0.54 μmole of PEP, 30 μg of protein, and erythrose-4-*P* and phenylalanine as indicated. The initial velocity is expressed as in Fig. 2.

Fig. 4. Secondary plot of the data from Fig. 1 for graphical determination of K_i of enzyme for phenylalanine according to Dixon. The initial rate of DAHP synthesis in the presence of phenylalanine, v_i, is expressed as μmoles of DAHP formed per 10 min per 1 ml of reaction mixture.

Effect of phenylalanine on the thermal inactivation of enzyme

It was found earlier that phenylalanine, like many allosteric effectors, stabilizes the phenylalanine-sensitive DAHP synthase against the inactivating effect of heat[15]. As shown in Fig. 5 the heat inactivation of enzyme at 50° follows first-order kinetics. The first-order rate constant, k_1, of the inactivation process of enzyme may be expressed as

$$k_1 = \frac{1}{t} \ln \left(\frac{E_0}{E} \right) \qquad (1)$$



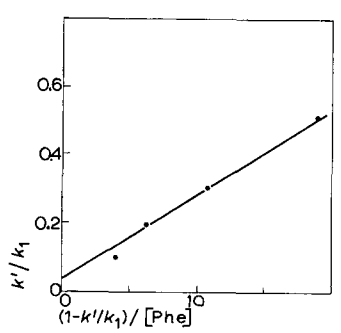


Fig. 5. Heat inactivation of DAHP synthase in the presence and absence of phenylalanine. 0.1 M Tris–HCl buffer (pH 7.5) containing 0.3 mg of protein and the indicated concentrations of phenylalanine was incubated at 50°. Samples were taken at time zero and the other times indicated and were diluted 10-fold to prevent reactivation in the standard reaction mixture for the determination of enzyme activity. The reaction mixtures were incubated at 37°, and the amount of DAHP formed was determined as described under MATERIALS AND METHODS.

Fig. 6. Secondary plot of the first-order rate constants k' and k_1 obtained from Fig. 5. The slope of the straight line from the plot of k'/k_1, *vs.* $(1 - k'/k_1)/[\mathrm{PHE}]$ according to Eqn. 2. is equal to K_i.

where E_0 and E are the concentration of the active enzyme at zero time and time t, respectively. The formation of enzyme–inhibitor complex is a bimolecular reaction but if the system contains a large excess of phenylalanine, the heat inactivation process follows apparent first-order kinetics (Fig. 5). The apparent first-order rate constant in this case, k', can also be calculated from Eqn. 1. From the first-order rate constants obtained it is possible to calculate the dissociation constant, K_i, of the phenylalanine–enzyme complex according to Eqn. 2 (ref. 16).

$$\frac{k'}{k_1} = \frac{K_i}{[\text{Phe}]}\left(1 - \frac{k'}{k_1}\right) + \frac{k_2}{k_1} \tag{2}$$

The plot of k'/k_1 *vs.* $(1 - k'/k_1)/[\text{Phe}]$ gives a straight line (Fig. 6), and the slope of this line is equal to K_i. With this method the value of K_i was also determined at other temperatures, and as shown in Table I, it is not temperature dependent. It is important to note that a similar analysis of the temperature dependence of the K_s of enzyme for its first substrate, PEP, gave quite the opposite result[9].

TABLE I

K_i OF ENZYME FOR PHENYLALANINE AT DIFFERENT TEMPERATURES

The experiments were carried out as described in Fig. 5.

Temp.	K_i *(μM)*
40°	36
50°	40
55°	37
60°	41

Inactivating effect of bromopyruvate

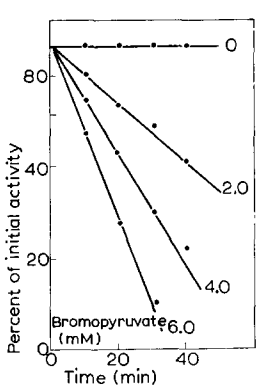
It was found previously that bromopyruvate, a structural analog alkylating agent of PEP, irreversibly inactivates the phenylalanine-sensitive DAHP synthase and PEP specifically protects the enzyme against the inactivation[17]. Although the reaction between the enzyme and bromopyruvate is bimolecular, in the presence of a large excess of bromopyruvate, the inactivation follows apparent first-order kinetics (Fig. 7). If bromopyruvate is a true substrate analog, then the first step of bromopyruvate–enzyme interaction might be the formation of an enzyme–bromopyruvate complex, E–bromopyruvate, which is transformed in a second step to the inactive alkylated enzyme, E_{alkyl}, and inorganic bromine.

$$E + \text{bromopyruvate} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\text{–bromopyruvate} \overset{k_2}{\rightarrow} E_{\text{alkyl}} + \text{Br} \tag{3}$$

The steady-state treatment of the reaction pathway described in Eqn. 3 according to MELOCHE[18] gives the rate equation

$$v_i = \frac{V_i}{\dfrac{K_i}{[\text{Bromopyruvate}]} + 1} \tag{4}$$

where v_i and V_i are the initial rates of inactivation of enzyme in the presence of finite



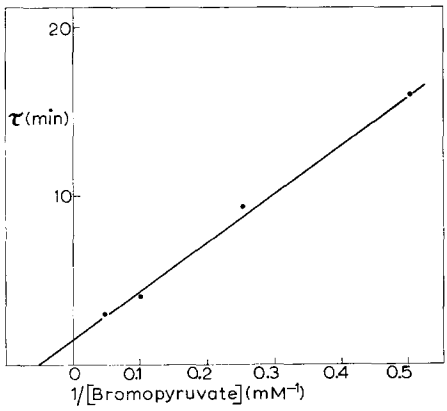


Fig. 7. Inactivation of DAHP synthase by bromopyruvate. 0.1 M potassium phosphate buffer (pH 7.5) containing 1 mg of protein per ml and the indicated concentrations of bromopyruvate was incubated at 22°. Samples were taken at zero time and other times as required and diluted 50-fold in the standard reaction mixture for determination of enzyme activity as described under MATERIALS AND METHODS.

Fig. 8. Inactivation half-time of DAHP synthase as a function of bromopyruvate concentration. Secondary plot (in part) of the data obtained from Fig. 7. according to Eqn. 5.

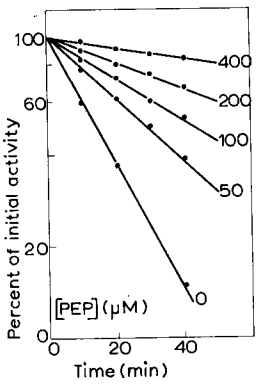
and infinite concentrations of bromopyruvate, respectively; K_i is $(k_1 + k_2)/k_1$ and represents the bromopyruvate concentration giving the half-maximum rate of inactivation and probably half-saturation of enzyme. The first-order rate constant of the overall inactivation process, k, shown in Fig. 7, can be calculated according to Eqn. 1. The inactivation half-time, τ (the time required for a given concentration of bromopyruvate to cause 50% inactivation of enzyme kinetically) is $\ln 2/k$; thus $\tau \propto 1/v_i$ and $T \propto 1/V_i$. After substitution of τ and T in Eqn. 4 its linear form will be

$$\tau = \frac{1}{[\text{Bromopyruvate}]}(TK_i) + T \qquad (5)$$

and the plot of τ *vs.* 1/[Bromopyruvate] according to Eqn. 5 should yield a straight line (Fig. 8). The intercept of this line on the ordinate is equal to T. The straight line obtained in Fig. 8 gives kinetic evidence that the initial step of the inactivation of enzyme with bromopyruvate is the formation of enzyme–bromopyruvate complex, and this initial step obeys the regular saturation kinetics. The inactivation half-time, T, from Fig. 8 is 1.5 min and the K_i of enzyme for bromopyruvate is 20 mM. As it appears from Fig. 9, PEP protects the enzyme against the inactivating effect of bromopyruvate and the extent of protection depends on the relation of concentration of PEP and bromopyruvate. If bromopyruvate and PEP are bound to the same site of enzyme, then the relation between bromopyruvate and PEP must be competitive. As was shown previously, DAHP synthase acts by a ping-pong mechanism, and the first substrate of the enzyme is PEP (ref. 9).

$$E + \text{PEP} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E\text{–PEP} \overset{k_4}{\rightarrow} E' + P_i \qquad (6)$$

In the presence of bromopyruvate and PEP all the intermediates described in Eqns. 3



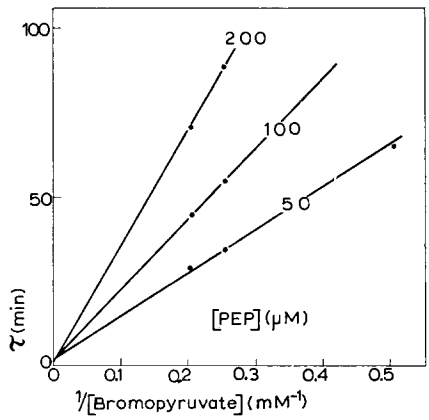


Fig. 9. Inactivation of DAHP synthase by bromopyruvate in the presence of PEP. Experimental conditions are the same as in Fig. 7 except that the reaction mixtures contained 4 mM bromopyruvate and the indicated concentrations of PEP.

Fig. 10. Inactivation half-time of DAHP synthase as a function of bromopyruvate concentration in the presence of PEP. Experimental conditions as described in Fig. 9.

and 6 are present in the reaction mixture. The steady-state treatment for the reaction pathways described in Eqns. 3 and 6 leads to the rate, which is defined by Eqn. 7 as described by MELOCHE[18]

$$v_i = \frac{V_i}{\frac{K_i}{[\text{Bromopyruvate}]} + \frac{K_1}{[\text{Bromopyruvate}]}\frac{[S]}{K_s} + 1} \tag{7}$$

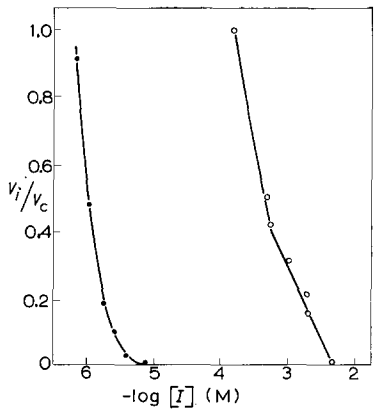
where K_m is $k_3/(k_3 + k_4)$. After substituting τ and T, the linear form of rate Eqn. 7 is

$$\tau = \frac{1}{[\text{Bromopyruvate}]} T\left(K_i + \frac{K_i[\text{PEP}]}{K_s}\right) + T \tag{8}$$

According to Eqn. 8 the plot of τ *vs.* 1/[Bromopyruvate] in the presence of different fixed concentrations of PEP gives a set of straight lines intersecting on the ordinate at the inactivation half-time, T. As shown in Fig. 10 the relation between PEP is competitive, as predicted from Eqn. 8. This means that bromopyruvate as substrate analog is bound to the same site as PEP. This result permits the specific labeling of the PEP binding site of the enzyme, using ^{14}C-labeled bromopyruvate, and the analysis of its amino acid sequence.

Inhibitory effect of sulfhydryl reagents

It was observed earlier that PHMB inhibits the activity of the tyrosine-sensitive DAHP synthase of *E. coli* and the DAHP synthase of *N. crassa*[4,5]. As Fig. 11 shows PHMB inhibits the activity of the phenylalanine-sensitive DAHP synthase as well. The inhibitory effect of PHMB is completely reversible. The activity of enzyme was fully regained by filtration on Sephadex G25 even after 24 h incubation at 0° in the presence of 40 μM PHMB. This result indicates that the binding of PHMB to the enzyme does not induce any irreversible secondary change in its conformation. Ell-



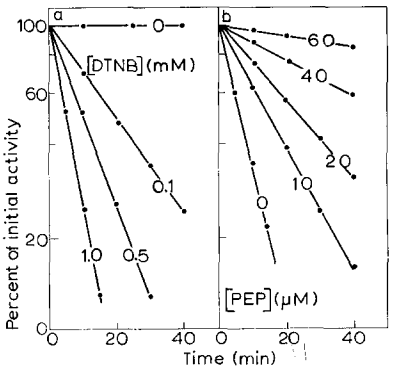


Fig. 11. Inhibition of the activity of DAHP synthase with PHMB and GSH. The reaction mixtures contained in a final volume of 1.0 ml, 50 μmoles of Tris–HCl buffer (pH 7.2), 30 μg of protein and PHMB (●—●) or GSH (○—○) as indicated. The mixtures were incubated at 0° for 20 min in the presence of glutathione, and for 10 min in the presence of PHMB. The inactivation reaction was arrested by the addition of substrates (0.5 μmole of PEP and 0.5 μmole of erythrose-4-*P* per ml), and the enzyme activity was determined as described under MATERIALS AND METHODS. V_i is the activity of the inhibited enzyme, and V_c is that of the control.

Fig. 12. Inactivation of DAHP synthase with DTNB and its protection by PEP. 0.1 M Tris–HCl buffer (pH 7.5) containing 0.4 mg of protein per ml and various concentrations of DTNB as indicated (a), or 1 mM of DTNB and various concentrations of PEP as indicated (b) were incubated at 22°. Samples were taken at zero time and other times as required and diluted 10-fold in the standard reaction mixture for determination of the residual enzyme activity.

man's reagent, DTNB, inactivates the enzyme. As demonstrated in Fig. 12a, in the presence of excess DTNB the inactivation process follows apparent first-order kinetics. It may be important from the point of view of the catalytic mechanism of enzyme action that PEP, the first substrate in the catalytic pathway, protects the enzyme against the inactivating effect of DTNB (Fig. 12b). The protective effect of PEP is specific because neither erythrose-4-*P*, the second substrate of the enzyme, nor phenylalanine, the allosteric inhibitor, has such an effect. In all probability the sulfhydryl groups reacting with DTNB are involved either in the PEP binding site(s) or in the catalytic site(s) of the enzyme.

The effect of metal-binding agents on the activity of enzyme

As described in the previous chapter, the phenylalanine-sensitive DAHP synthase is a sulfhydryl enzyme. On the basis of this finding, the observation that the activity of the enzyme is inhibited by thiol compounds like mercaptoethanol or GSH was rather unexpected. As Fig. 11 shows, 0.45 mM of GSH causes 50% inhibition of enzyme activity. Since thiol compounds are known as heavy-metal-binding agents, it was supposed that the phenylalanine-sensitive DAHP synthase contains bound heavy metal. Previous studies have shown that the activity of DAHP synthase of *N. crassa* and the tyrosine-sensitive isoenzyme of *E. coli* are inhibited by EDTA and cyanide[4,5]. After EDTA treatment these enzymes were slightly activated by Co^{2+}, and it was concluded that they are Co^{2+}-containing enzymes. Because of the observed inhibitory effect of thiol compounds it was reasonable to suppose that the phenylalanine-sensitive DAHP synthase may be similar to those described previ-

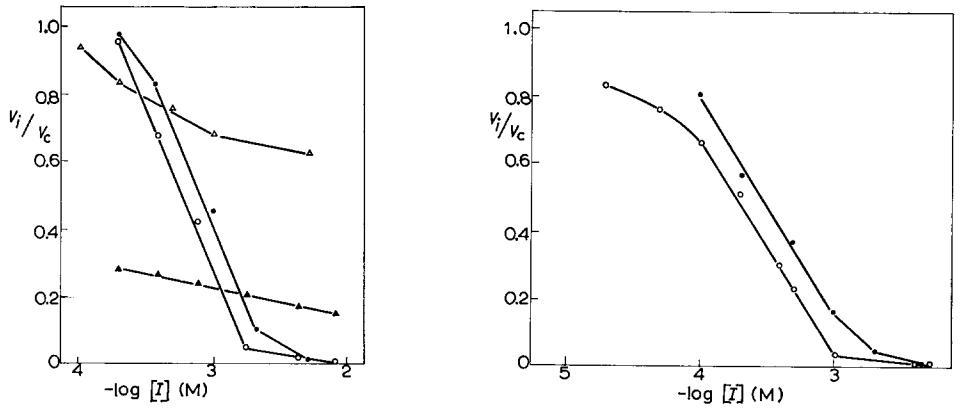


Fig. 13. Inhibitory effect of cyanide and EDTA on the activity of DAHP synthase. The enzyme, 30 μg per ml, in 0.05 M Tris–HCl buffer was incubated at 0° for 40 min, either with KCN (pH 8.0; ○—○); or cyanide (pH 7.2; ●—●); or EDTA (pH 8.0; ▲—▲); or EDTA (pH 7.2; △—△). Other experimental conditions were as described in Fig. 11.

Fig. 14. Inhibition of the activity of DAHP synthase with metal-binding agents at pH 7.2. Experimental conditions were as described in Fig. 13. ○—○, 1-nitroso-2-naphthol; ●—●, *o*-phenanthroline.

ously. As shown in Fig. 13, the activity of the phenylalanine-sensitive DAHP synthase is inhibited by EDTA and cyanide. The inhibitory effect of EDTA strongly depends on the pH of the incubation mixture. In spite of the fact that EDTA completely inhibits the activity of the enzyme, the inhibition is fully reversible. The EDTA-treated enzyme, even after 24 h of incubation at 0° is fully active after Sephadex G-25 filtration. These data support the conclusion that the heavy metal is a firmly bound component of the enzyme molecule and the reversible nature of inhibition is consistent with the formation of a dissociable enzyme–inhibitor complex. Although the inhibitory effect of cyanide is only slightly dependent on the pH of the medium (Fig. 13), the reversibility of its action is very dependent on the pH. At pH 7.2 the inhibitory effect of cyanide is reversible up to 1–2 h, but at pH 7.8 it is irreversible in minutes. Because EDTA and cyanide react with many different metal ions, it is difficult to make any statements concerning the nature of heavy metal bound to DAHP synthase on the basis of these experiments. Among the more specific chelating agents tested, the inhibitory effect of *o*-phenanthroline and 1-nitroso-2-naphthol are shown in Fig. 14. It has been reported that *o*-phenanthroline is not only a metal-binding agent but in the presence of trace amounts of Cu^{2+} it also causes the oxidation of sulfhydryl groups[19,20]. Since DAHP synthase is an sulfhydryl enzyme, it was important to know that the inhibitory effect of *o*-phenanthroline is concerned with its metal-binding or with its oxidizing property. The finding that the *o*-phenanthroline-treated inactive enzyme is fully active after Sephadex G-25 filtration and that the inhibition is completely reversible shows decisively that *o*-phenanthroline does not cause the oxidation of the enzyme, but it forms a dissociable enzyme–inhibitor complex. 1-Nitroso-2-naphthol is a rather specific reagent for Co^{2+}, but it also reacts with Fe^{2+}. It was found that α,α'-dipyridyl, a specific reagent for Fe^{2+}, does not influence the activity of enzyme, and the possibility that the phenylalanine-sensitive

DAHP synthase contains Fe^{2+} was rejected. It is known that 1-nitroso-2-naphthol initially acts on Co^{2+} by oxidizing the ion to Co^{3+}, after which it forms a chelate with the Co^{3+} formed. If the isoenzyme 1a of DAHP synthase is a Co^{2+} enzyme, the inhibition of its activity with 1-nitroso-2-naphthol must be irreversible. It was found that 1-nitroso-2-naphthol indeed irreversibly inactivates the enzyme, as expected from its mechanism of action. This indirect evidence, together with the activating effect of Co^{2+} on the activity of enzyme, suggests that the DAHP synthase studied is a heavy metal enzyme containing Co^{2+}. At the present time it is not known if the bound Co^{2+} is only a structural component of the enzyme or if it plays some role in the catalytic process as well. Direct determination of the nature and amount of heavy metal in the phenylalanine-sensitive DAHP synthase is in progress.

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