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THE NATURE OF 3-DEOXY-D-*ARABINO*-HEPTULOSONATE 7-PHOSPHATE SYNTHASE IN EXTRACTS OF WILD-TYPE *NEUROSPORA CRASSA*: A REACTION CONTROLLED BY TWO ACTIVATING SUBSTRATES AND THREE ALLOSTERIC NEGATIVE MODIFIERS

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SUMMARY

1. The 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase (7-phospho-2-keto-3-deoxy-D-*arabino*-heponate D-erythrose-4-phosphate-lyase (pyruvate-phosphorylating), EC 4.1.2.15) (DAHP synthase) of *Neurospora crassa* wild type 74A is a unit of physiological function but regulatory differentiation. Kinetic measurements are made with crude extracts in order to minimise denaturation of possible unifying native molecular organisation. A number of tentative conclusions (see below) are reached regarding ligand-enzyme interactions.

2. DAHP synthase is a thiol enzyme. Co^{2+} stimulates variably, EDTA inhibits strongly and is reversed completely by Co^{2+}. Between pH 6.2–8.0 there is little change from an optimal activity at about pH 6.5. An Arrhenius plot is linear without transition between 17° and 37°. Heat denaturation occurs above about 41°. ΔH^* is approx. 16 000 cal/mole. Crude dialysed extracts are stable for at least 1 h at 37°, but are rapidly deactivated by dilution.

3. The substrates erythrose 4-phosphate and phosphoenol pyruvate are also activators (positive modifiers) with strong cooperative interaction between a minimum of 2 active sites per substrate species ($m = 1.7$–1.8). Apparent K_m does not apply in these examples, but $[S]_{0.5(V)}$ for either substrate is about $1.8 \cdot 10^{-4}$ M. The reaction is presumptive ping-pong but includes cooperative site interactions. It is deduced that PEP adds first and the importance of PEP as an initiating metabolite of aromatic biosynthesis is discussed.

4. At least three distinct kinds of active sites occur; those inhibited by phenylalanine, those inhibited by tyrosine and those inhibited by tryptophan. The ratio between these is normally about 44:44:10. Inhibition by tryptophan has not previously been demonstrated at this site in Nature. These end products are negative modifiers each showing strong cooperative interaction between at least two sites. They act in part by lowering cooperation between substrate molecules. One molecule of tyrosine

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

may sometimes bind ($m = 0.9$, $[M]_{0.5} = 6 \cdot 10^{-6}$ M). The phenylalanine and tyrosine sites must partly interact since their inhibition pattern is synergistic. Inhibition by tyrosine and phenylalanine is noncompetitive with regard to each substrate. When $m = 1.4$ to 2, values of $[M]_{0.5}$ range from $8 \cdot 10^{-6}$ to $1.7 \cdot 10^{-5}$ for different negative modifiers.

The enzyme is rapidly denatured at 45°. The phenylalanine-sensitive component is most labile, but selectively protected by phenylalanine. The tyrosine-sensitive component is selectively protected by phosphoenol pyruvate (PEP). The tryptophan-sensitive portion and a small non-inhibited portion survive for at least 1 h without protection. Heat denaturation does not create a non-inhibited portion.

INTRODUCTION

3-Deoxy-*arabino*-heptulosonate 7-phosphate synthase (7-phospho-2-keto-3-deoxy-D-*arabino*-heptonate D-erythrose-4-phosphate-lyase (pyruvate-phosphorylating), EC 4.1.2.15) (DAHP synthase) is the first enzymic function unique to the aromatic path of microorganisms. In the procaryotes *Escherichia coli* and *Bacillus subtilis* it has been shown to be a site for control of the path[1]. This is done in *E. coli* by feedback inhibition and repression, with the aromatic amino acids, tyrosine, phenylalanine and tryptophan as the metabolic effectors. At least three isoenzymes exist and participate in regulation. By contrast, in *B. subtilis* it is chorismic acid and prephenic acid(see

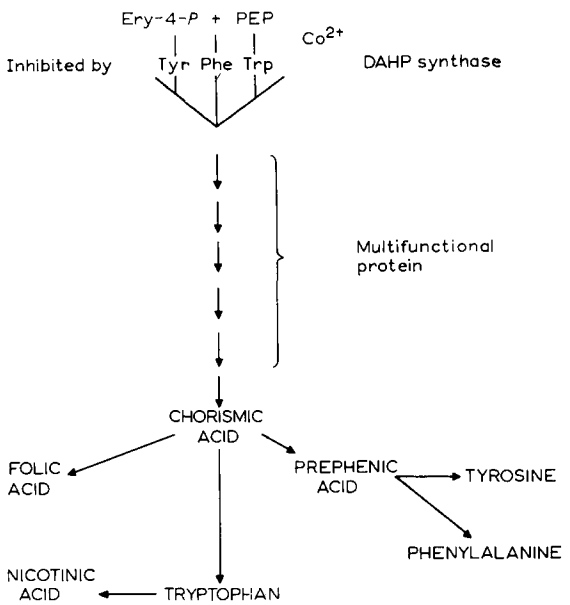


Fig. 1. Outline of aromatic biosynthesis in *N. crassa*. In general, intermediates are the same as for *E. coli*, only key intermediates and end products have been given. Of the several vitamin end products of the pathway in *E. coli* only 4-aminobenzoate (folic acid branch) has been established in Neurospora. Unlike *E. coli* nicotinic acid is derived from tryptophan.

Fig. 1) that are the feedback inhibitors and these modify a single enzyme rather than isoenzymes.

It was considered desirable to commence a detailed study of this potential site of regulation and functional organisation in a eucaryote in which biochemical studies could still be readily complemented by genetic investigation. The organism selected was the fungus *Neurospora crassa*.

It is recognised increasingly that biosynthetic functions are frequently organised within the cell as multifunctional proteins which may have a sub-unit structure made up of the same or differing polypeptides. Evidence that this applies to the reactions of the aromatic path is accumulating rapidly[1]. It has been pointed out[2] that the recognition of organisation is facilitated when function is so dependent on the native organisation that the effects of sub-unit disaggregation or of specific mutations, obviously affect multiple functions. If sub-units readily dissociate and retain individual functions when separate or when combined with defective sub-units, the recognition of native association may pass unnoticed.

In Neurospora it is known that the five enzymic functions subsequent to DAHP synthase constitute a unit of organisation[3,4] as do at least three of the tryptophan path functions[5,6]. The pre-chorismate biosynthetic functions are held in common by all the aromatic end products (Fig. 1). Molecules synthesised by these reactions are not differentiated with respect to destination until after chorismate. Thus, from the gross physiological point of view it is the total synthesis of DAHP that matters, not which molecules were made by active sites controlled by one specific aromatic path end product or another. The differentiation of DAHP synthase into operational units controlled by specific allosteric end products is one way of ensuring that the synthesis of chorismate is adjusted to the total requirements of the individual pathways branching at chorismate.

In the present paper the properties of DAHP synthase are examined in dialysed crude extracts and treated as a physiological unit which in the native state might constitute a physical unit of biosynthetic function and regulation. It will become apparent that the enzyme has readily identifiable components concerned with the regulation of function. Subsequent publications will describe isoenzymic components that appear upon "purification" and examine the biochemical genetics of these components[7–9].

A casual laboratory acquaintance with the DAHP synthase of *E. coli* and *N. crassa* makes it obvious that even simple manipulations lead to far-reaching changes in the character of the enzyme, especially with regard to the characteristics of allosteric inhibition. Interpretations based on these altered characteristics clearly do not relate to the native state and the physiological needs of the organism. Thus, although it is usual to present kinetic data on highly purified preparations, the present intention is to present data and interpretations as a basis for further studies. Every attempt has been made to ensure the reliability of the kinetic data but because of the use of crude extracts the conclusions presented must be regarded as tentative, providing working hypotheses for future experimentation. Some of the results included in this paper have been reported in preliminary form[10–12].

Investigations concerning the interactions between DAHP synthase and aromatic amino acids added to the exogenous environment will be reported separately[13].

MATERIALS AND METHODS

Organism. Wild type *N. crassa* 74A was kindly provided by Professor N. GILES and Dr. MARY CASE. DAHP was generously provided by Dr. D. B. SPRINSON.

Culture. Basic stocks were kept on silica gel and working cultures on VOGEL's minimal medium[14] solidified with 1% Oxoid Ion agar. Material for making extracts was grown in VOGEL's minimal liquid medium (1 l) in 2-l conical flasks shaken on a Gyrotory shaker for 2.5 days at 25°. The mycelium was harvested by filtration and well washed with distilled water.

Extracts. Extracts were prepared from mycelium (usually freshly harvested, but sometimes after storage in the deep freeze) by grinding with glass powder and 0.1 M phosphate buffer (pH 6.4), cooled in ice. Mycelium (wet) and buffer were taken in the approx. ratio of 1:2 by weight. Debris was removed by centrifugation at approx. 22 000 × *g* for 20 min. Protein[15] was 12–17 mg per ml. Extracts were dialysed for 3 × 1 h against 0.05 M phosphate buffer (pH 6.4). Wherever possible extracts were used immediately, but otherwise were kept at −15° and carefully thawed before use.

The measurement of DAHP synthase. The method was basically that reported elsewhere using the cyclohexanone extraction procedure[16]. The substrates D-erythrose 4-phosphate (Ery-4-P) and phosphoenol pyruvate (PEP) were each $2 \cdot 10^{-3}$M unless used at non-saturating levels as indicated. Buffer was 0.05 M KH_2PO_4–NaOH (pH 6.4) unless varied as indicated. Total volume was 0.25 ml, reaction was at 37° for 10 min. Absorbance in the final 3 ml of cyclohexanone has been correlated with molarity. With the sample size taken, $A_{549\ m\mu} = 0.53$ corresponded to 10^{-4} M DAHP in the reaction mixture. Absorbance was proportional to concentration from 10^{-6} M to at least 10^{-3} M. An extract of 74A prepared as above had a specific activity of $A_{549\ m\mu}$ 1.8–2.5 per mg protein per ml reaction mixture in 10 min. For the present paper, conversion to molarity was not required. An $A_{549\ m\mu}$ of 0.53 represents a 5% change based on saturating substrates and $A_{549\ m\mu}$ 0.011 represents $2 \cdot 10^{-6}$ M.

For kinetic measurements, extract was added by microsyringes. Solutions of variables were diluted as required to add them always at the same volume. In order that this study be possible with the facilities available, DAHP synthase was measured routinely at only one time (10 min) as has been usual for work with this enzyme in various laboratories. This is necessitated by the tediousness of the assay procedure. It was demonstrated that with excess substrates the reaction rate was linear from zero to at least 20 min and proportional to extract with the amount used (about 0.15 mg protein). With the nonsaturating levels used in titration experiments the 10-min sample approximated to the zero time rate when checks were done. Checks indicated a standard deviation of from 4 to 4.5% and a standard error of from 2 to 2.3%.

Nomenclature. The author has recently pointed out that the apparent K_m values obtained by extrapolation of curved double reciprocal plots do not apply[12]. Since then the remarks of ATKINSON[17] following a suggestion of KOSHLAND, NEMETHY AND FILMER[18] have been seen and the nomenclature suggested has been adopted.

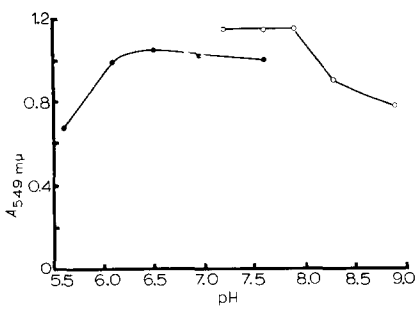
Kinetics. Where S = substrate and M = modifier, a small molecule interacting with the enzyme and altering its catalytic behavior, then, $[S]_{0.5\,(V)}$ or $[M]_{0.5\,(V)}$ is used to designate the concn. required for half saturation in terms of the reaction velocity. The Hill equation in the form

$$\log [v/(V - v)] = n \log [S] - \log K$$

where v is reaction velocity, V is the maximum reaction velocity, n is the number of sites at which S may bind and K is a constant, is often used for the estimation of n from the slope of the plot, $\log[v/(V-v)]$ against $\log[S]$. In this paper, m, rather than n, is used to designate the slope since this more accurately depicts a value which is really a function of n and the strength of interaction between sites. In the example of negative modifiers a similar relationship was obtained by plotting $\log[(V_0-v)/(v-V_{sat})]$ against $\log[M]$ where V_0 is the rate in the absence of modifier and V_{sat} is the rate in the presence of saturating modifier. Because the molecular organisation of DAHP synthase is still unknown the particular value taken for V_{sat} has been varied as indicated in the text and tables.

RESULTS

In preliminary studies 0.05 M phosphate buffer (pH 6.4) was used[10–12,19]. Under pH 6.0 activity falls off steeply. A titration of activity between pH 6.0 and 9.0 using phosphate and Tris buffers showed a plateau between pH 6.2 and pH 8.0 (Fig. 2).



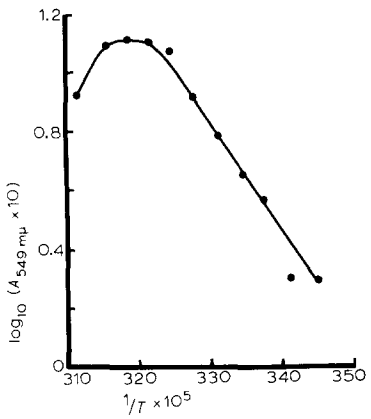


Fig. 2. Activity of DAHP synthase at different pH values. $A_{549\ m\mu}$ is proportional to specific activity. ●, phosphate buffers; ○, Tris buffers, pH values at 37°.

Fig. 3. Arrhenius plot for DAHP synthase. Range 17° to 50°, above about 38° conditions are sub-optimal. $A_{549\ m\mu}$ is proportional to the initial velocity. The energy of activation E is 16 600 cal per mole, therefore ΔH^* is 16 000 cal/mole.

Activity in Tris buffer was slightly better than in phosphate buffer. There was some effect of phosphate (a reaction product) on the enzyme since an increase in phosphate concn. to 0.5 M decreased activity. However there was a greater degree of instability in Tris (Table I). For this reason all experiments reported in this paper were done with 0.05 M phosphate buffer pH 6.4–6.6. Dialysis in this buffer (see METHODS) increased activity presumably by removing endogenous inhibitors.

Cobalt as a co-factor. Cobalt often stimulated crude extracts and completely reversed inhibition by EDTA (Table II). Mg^{2+} did not behave in this manner. In the present study Co^{2+} was not added, as the effect of these ions was not great unless

TABLE I

ACTIVITY OF DAHP SYNTHASE AFTER DILUTION AND HEATING AT 37° IN PHOSPHATE AND TRIS BUFFERS

Activity is given as $A_{549\ m\mu}$. A control was estimated in the usual manner using 0.01 ml extract (see MATERIALS AND METHODS); activity, 0.94. In other examples a 1 in 10 dilution of dialysed crude extract was made into buffer, 0.5 M, preheated to 37°; zero time samples (0. ml) were withdrawn immediately, others as indicated. Buffer in the samples was used to buffer reaction mixtures (for the effect of pH on reaction see Fig. 2). Protein was the same in all examples (about 0.1 mg).

Time at 37° after dilution (min)	*Phosphate buffer*			*Tris buffer*			
	pH 6.1	*6.9*	*7.6*	*7.2*	*7.6*	*7.9*	*8.9*
0	0.525	0.52	0.53	0.675	0.58	0.64	0.375
5	0.565	0.645	0.49	0.27	0.225	0.255	0.13
15	0.305	0.56	0.45	0.21	0.215	0.2	0.07

extracts were first treated with EDTA. The effects of Co^{2+} are more complex than the data of Table II indicate and are being further investigated.

Enzyme stability. DAHP synthase was unstable during manipulations such as dilution before assay, ammonium sulphate fractionation, chromatography on DEAE cellulose, molecular sieving etc. The problem of instability does not affect the work reported in this paper since undiluted extracts were used, or conditions that are now known to greatly stabilise the enzyme[8]. Iodoacetate did not stabilise the enzyme, or deactivate it, but *p*-chloromercuribenzoate (10^{-4} M) completely inhibited activity, indicating the importance of thiol groups to function. Mercaptoethanol and dithiothreitol (Cleland reagent) may have had a slight protective action against dilution

TABLE II

COBALT AS A COFACTOR FOR DAHP SYNTHASE

A crude dialysed extract (0.01 ml) was used. $A_{549\ m\mu}$ is proportional to activity. $CoCl_2$ at the molarity indicated was present during the enzyme reaction. For pretreatment, EDTA was added to extract (0.5 ml) to give either $2.5 \cdot 10^{-3}$ M or 10^{-2} M and held on ice for 30 min. EDTA present during subsequent enzymic reaction was therefore 10^{-4} M and $4 \cdot 10^{-4}$ M, respectively (since 0.25 ml reaction mixture contains 0.01 ml extract).

Pretreatment	*$CoCl_2$ (mM)*	*$A_{549\ m\mu}$*
None	—	1.514
None	0.1	1.7
None	1.0	1.79
2.5 mM EDTA	—	0.434
2.5 mM EDTA	0.1	1.79
2.5 mM EDTA	1.0	1.79
2.5 mM EDTA	—*	0.342
10 mM EDTA	—	0.287
10 mM EDTA	1.0	1.79

* In this case 1.0 mM $MgCl_2$ was present instead of $CoCl_2$.

denaturation. Above 10^{-4} M these reagents inhibited the chemical assay for DAHP presumably by reacting with periodate.

Temperature optimum. An Arrhenius plot is given in Fig. 3 and approximates to linearity from the lowest temperature tested, 17°, to just above 37°. Higher than this, activity was affected by what is probably heat denaturation (see later). Continuity of linearity lends support to the assumption that if different kinds of DAHP synthase active sites exist they have similar activities in the absence of specific negative modifiers. The energy of activation calculated from this data is 16 580 cal and therefore ΔH^* is about 16 000 cal (see SPRINSON[20]). This is considerably higher than the values reported for DAHP synthase from *B. subtilis*[21]. A reaction temperature of 37° was used in the work reported in this paper. Although close to the temperature where heat deactivation may begin, experiments have indicated that the undiluted extract (12–17 mg protein) is stable for at least 1 h at 37°.

Interactions between DAHP synthase and its ligands

Substrate–enzyme interactions. The substrates are PEP and Ery-4-*P*. When one substrate was varied in the presence of an excess of the other, the reciprocal plots of $1/v$ against $1/[S]$ gave curves rather than straight lines (Figs. 4 and 5). These have the

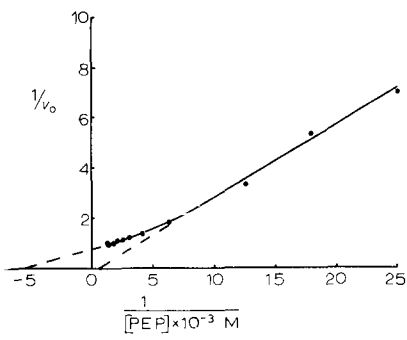


Fig. 4. DAHP synthase, double-reciprocal plot of initial velocity, $v_0(A_{549\ m\mu})$ with varying PEP and saturating Ery-4-*P* concn. ($2 \cdot 10^{-3}$ M). The broken lines are extrapolations of the asymptotes.

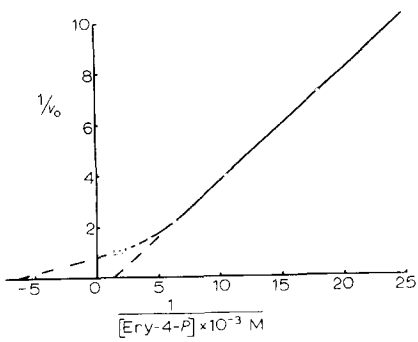


Fig. 5. DAHP synthase, double-reciprocal plot of initial velocity, $v_0(A_{549\ m\mu})$ with varying Ery-4-*P* and saturating PEP concn. ($2 \cdot 10^{-3}$ M). The broken lines are extrapolations of the asymptotes.

form of non-rectangular hyperbolas. Whenever the curved portions have been titrated in detail and then replotted by substituting $1/[S]^2$ for $1/[S]$, an approximation to a straight line has been obtained (see also Fig. 11). This second order response is indicative that for both substrates at least two sites are involved in the reaction. The non-rectangular hyperbolic form is consistent with the view that DAHP is formed as the result of breakdown of both *ES* and *ESS* complexes (Fig. 12).

That the binding of one molecule of substrate increases the "affinity" of the enzyme for another is shown from values of $m = 1.8$ for Ery-4-*P* and $m = 1.7$ for PEP where m is a function of n the number of sites and the strength of interactions between

TABLE III

INDEX OF INTERSITE COOPERATION (m) AND LIGAND CONCENTRATION AT HALF SATURATION ($[S]_{0.5}$ or $[M]_{0.5}$)

The derivation of m, $[S]_{0.5}$ and $[M]_{0.5}$ is given in MATERIALS AND METHODS. $[S]_{0.5}$ applies to the substrates PEP and Ery-4-*P* and $[M]_{0.5}$ to the negative modifiers phenylalanine, tyrosine and tryptophan.

Ligand	*Source of data*	m	$[S]_{0.5}$ *or* $[M]_{0.5}$ *(M)*
PEP	Figs. 4, 10	1.15*, 1.7	$1.8 \cdot 10^{-4}$
PEP in presence Phe	Fig. 10	1.1	1.4 to $1.5 \cdot 10^{-4}$ see text
PEP in presence Tyr	Fig. 10	1.15	
PEP in presence Phe + Tyr	Fig. 10	0.85	
Ery-4-*P*	Figs. 5, 9	1.8	$1.8 \cdot 10^{-4}$
Ery-4-*P* in presence Phe	Fig. 9	1.1	
Ery-4-*P* in presence Tyr	Fig. 9	1.4	
Ery-4-*P* in presence Phe + Tyr	Fig. 9	1.0	
Phenylalanine	Fig. 8	2.4 (2.0)	$1.7 \cdot 10^{-5}$
	see text	1.7** (1.4)**	$1.8 \cdot 10^{-5}$
Tyrosine	Fig. 8	2.3 (1.7)	$1.5 \cdot 10^{-5}$
	see text	1.45** (0.9)**	$6 \cdot 10^{-6}$
Phenylalanine + Tyr	Fig. 8	1.7	$1.2 \cdot 10^{-5}$
	see text	1.5**	$1.0 \cdot 10^{-5}$
Tryptophan	Fig. 11	2.0 (1.8)	$8 \cdot 10^{-6}$ to 10^{-5}

* The value m applies to PEP at low concentration up to about $8 \cdot 10^{-5}$ M. Values of m in brackets refer, in the examples of phenylalanine and tyrosine, to calculations using a v_{max} value including total activity inhibited by phenylalanine plus tyrosine; in the example of tryptophan, to a v_{max} value ignoring the non-inhibited activity.

** This data refers to an extract for which only one molecule of tyrosine reacted with the enzyme (see text).

them (for data see Figs. 4 and 5 and Table III). Thus there is homomolecular cooperation in a manner which is usually recognised as necessitating conformational changes. The m values quoted indicate that at least two sites exist for the binding of

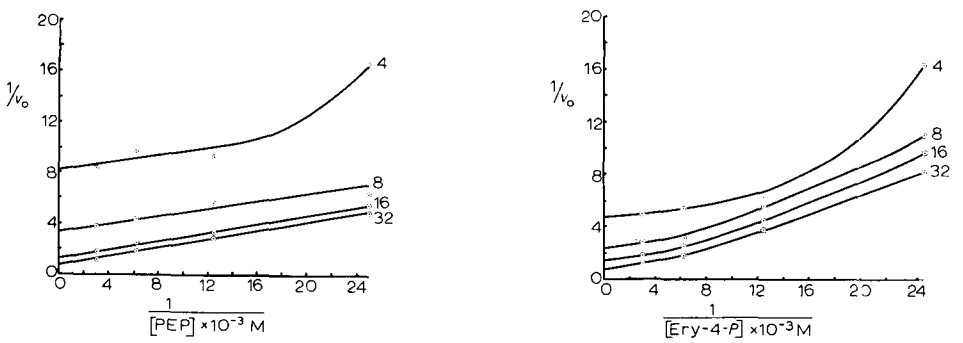


Fig. 6. DAHP synthase, double reciprocal plot of initial velocity $v_0(A_{549\ m\mu})$ with varying PEP at four fixed concentrations of Ery-4-*P* as indicated ($\times 10^{-5}$ M).

Fig. 7. DAHP synthase, double reciprocal plot of initial velocity $v_0(A_{549\ m\mu})$ with varying Ery-4-*P* at four fixed concentrations of PEP as indicated ($\times 10^{-5}$ M).

each substrate. Kinetic constants have not been calculated since K_m is not considered applicable in these examples[12]. Following other usage[17,18], $[S]_{0.5(V)}$ is about $1.8 \cdot 10^{-4}$ M for Ery-4-*P* and $1.8 \cdot 10^{-4}$ M for PEP.

These various interpretations depend on the assumption that in the absence of allosteric modifiers DAHP synthase has the same or very nearly the same catalytic properties, irrespective of potential subunit character. None of the evidence is inconsistent with this assumption.

In other experiments one substrate was varied with the other held constant, but non-saturating. According to CLELAND[22] a family of $1/v$ against $1/[S]$ plots, if parallel, usually indicates a ping-pong reaction; that is, release of the first product occurs before the addition of the second substrate and the enzyme has two stable forms (Fig. 12). The results in general took this form (Figs. 6 and 7). In the example of PEP (Fig. 6),

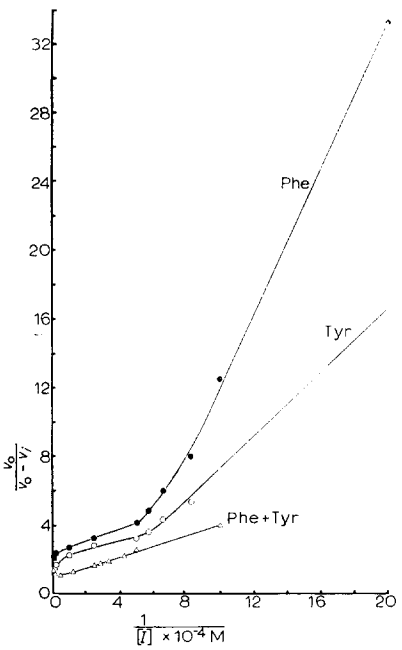


Fig. 8. Double reciprocal plots of the inhibition of DAHP synthase. Both substrates saturating ($2 \cdot 10^{-3}$ M). In the experiments with phenylalanine plus tyrosine an equimolar mixture was used and plotted as total molarity. ●, phenylalanine; ○, tyrosine and △, phenylalanine *plus* tyrosine.

one point, corresponding to the lowest concentration of both substrates, diverged (3 experiments). When Ery-4-*P* was titrated all the plots were parallel, but curving (Fig. 7). Curvature is taken as evidence that cooperative effects are superimposed on the ping-pong mechanism.

Inhibitor–enzyme interactions

Inhibition by phenylalanine and tyrosine. Portions of DAHP synthase were inhibited by phenylalanine, by tyrosine or the two in equimolar mixtures (Fig. 8). On the basis of total molarity the mixtures are a better inhibitor than the sum of the

parts. In double reciprocal plots the individual negative modifiers usually gave non-rectangular hyperbolas similar to the results with substrates (Figs. 4 and 5). The cooperation between phenylalanine and tyrosine was shown by strong inhibition and linearity throughout the range studied. The enhanced inhibition in the presence of both modifiers is particularly evident in *v versus* [*S*] plots. It appears that separate active sites are primarily sensitive to phenylalanine and tyrosine respectively, but that a concerted modifier effect can be transmitted between the different species of site to produce synergism. This demands a physical association between the sites. For at least one extract, tyrosine has reacted in a noncooperative manner (Table III). Velocity *versus* substrate concentration plots still indicate synergism between phenylalanine and tyrosine, but this is weaker.

At very high concentration, single molecular species gave downward curves while the mixture gave an upward one (Fig. 8). Presumably under these conditions each modifier is recognised at sites normally recognising the other but when both modifiers are present this cross reaction is prevented and there is some mutual interferenze.

Values of m have been calculated from the above data using V_{sat} derived from the portions of activity sensitive to one inhibitor or to both (Table III). By considering the phenylalanine- and tyrosine-sensitive portions as a functional unit, m values suggesting a minimum of two interacting sites for each ligand were obtained, giving a one to one ratio between active and inhibitor sites and their interactions. Considering the phenylalanine- and tyrosine-sensitive portions independently, m exceeds 2 in some examples. On this basis the number of sites might be 4 in which event cooperation is only moderate.

A series of substrate titrations were done in the presence of fixed, non-saturating amounts of the negative modifiers. In each variation inhibition occurred throughout the titration of Ery-4-*P* (Fig. 9) but a threshold level of PEP was required before inhibition became significant (Fig. 10). This threshold coincides with the change from

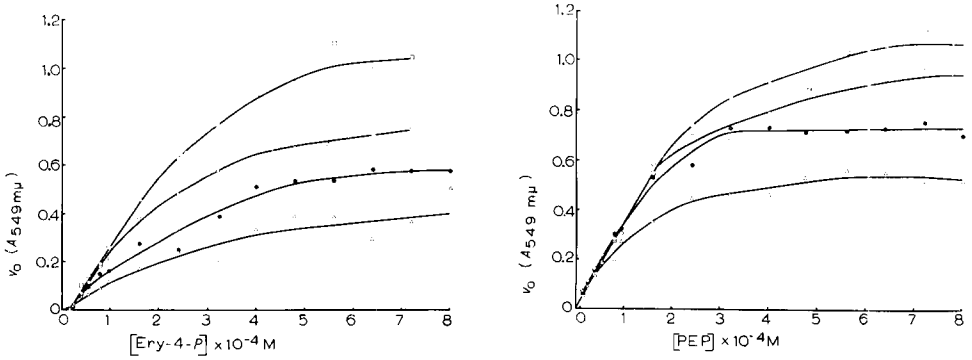


Fig. 9. DAHP synthase, titration of Ery-4-*P* with PEP in excess ($2 \cdot 10^{-3}$ M) with and without inhibiting ligands. □, no inhibitors; ●, *plus* phenylalanine $2 \cdot 10^{-5}$ M; ○, *plus* tyrosine $7.5 \cdot 10^{-6}$ M and △, *plus* phenylalanine and tyrosine each 10^{-5} M.

Fig. 10. DAHP synthase titration of PEP with Ery-4-*P* in excess ($2 \cdot 10^{-3}$ M) with and without inhibiting ligands. □, no inhibitors; ●, *plus* phenylalanine $2 \cdot 10^{-5}$ M; ○, *plus* tyrosine $7.5 \cdot 10^{-6}$ M and △, *plus* phenylalanine and tyrosine each 10^{-5} M.

non-cooperative to cooperative binding, (Table III), suggesting that the latter is required before inhibition is possible. In the presence of the inhibitors, m values for substrates were lowered (Table III), suggesting that the inhibitors antagonise co-operation between the substrate sites. The maximum velocity was lowered also and for Ery-4-P, $[S]_{0.5(V)}$ was clearly unaltered. For PEP in the presence of inhibitors, $[S]_{0.5(V)}$ is more difficult to assess. If DAHP formed during the non-cooperative, non-inhibited phase is ignored, the values range from $1.4–1.6 \cdot 10^{-4}$ M for the variables. That v_{max} alters, but $[S]_{0.5(V)}$ does not, is a non-competitive type of inhibition. This was also indicated when double reciprocal plots were made.

Inhibition by tryptophan. This portion of activity is normally a minor component and cannot be measured accurately in the absence of phenylalanine and tyrosine[19]. However, in contrast to the phenylalanine and tyrosine inhibited portions it is stable during manipulations and to moderate heat (see below). The characteristics of inhibition by tryptophan, after destroying other inhibited activity by heat, are given in

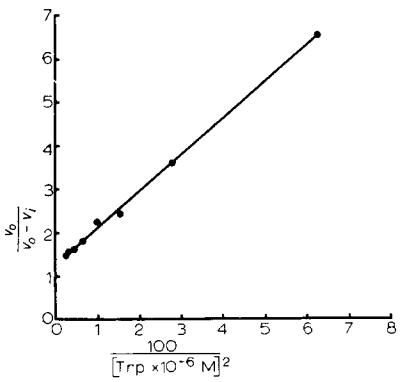


Fig. 11. Inhibition of heat-stable DAHP synthase by tryptophan. Crude dialysed extract was heated at 45° for 10 min then 0.03 ml (three times the amount usually taken) was used for each estimation of DAHP activity. If the non-inhibited portion of activity (about 23% of the total) is ignored in evaluating $v_0/v_0 - v_i$, linearity is retained, but with lessened slope beneath the plot given.

Fig. 11. This double reciprocal plot emphasises the second order kinetics of inhibition. The total DAHP synthase activity remaining after heating has been used (V_0) and this includes about 23% that is not inhibited. If this portion is ignored a linear plot is still obtained but with lessened slope. The corresponding values of m are given in Table III.

All the other organic ligands of DAHP synthase (Ery-4-P, PEP, phenylalanine and tyrosine) give similar plots to Fig. 11 when data corresponding to the curved portions of the more usual double reciprocal plots are re-plotted.

Heat deactivation in the absence and presence of various ligands. Dilution of dialysed extracts before measuring activity results in the loss of 80–90% activity within a few minutes. The activity in undiluted extracts containing 12–17 mg/ml protein was relatively stable at room temperature and 37°, retaining full activity after 1 h at 37° but was unstable at 45°. Ery-4-P gave no protection against this deactivation, but PEP gave considerable protection. The pattern of protection by PEP and phenyl-

TABLE IV

DIFFERENTIAL STABILITY AT 45° OF PORTIONS OF DAHP SYNTHASE INHIBITED BY DIFFERENT AROMATIC AMINO ACIDS

Activity is normalised to the value 100 given to the non-heated control. Ligands when added were at 10^{-3} M. Phenylalanine and tyrosine were removed by dialysis for 2 × 1 h against phosphate buffer 0.05 M (pH 6.4).

Conditions of heating at 45°	*Relative activity*						
	Total	*Inhibited by*					*Not inhibited*
		Phe	*Tyr*	*Phe + Tyr*	*Trp*	*Phe + Tyr + Trp*	
Not heated	100	48	43	82	14	97	3
10 min	16	0	0	0	14	14	2
10 min + Ery-4-*P*	16	0	0	0	14	14	2
10 min + PEP	60	12	31	44	13	57	3
10 min + Phe	70	52	11	54	20	65	5
60 min + PEP	32	0	16	16	13	30	2
60 min + PEP + Tyr	32	6	18	19	13	29	3

alanine is summarised in Table IV. The tryptophan-sensitive portion and a non-inhibited portion survived all treatments. PEP protected the tyrosine-sensitive portion selectively compared to the phenylalanine-sensitive portion. The latter was protected selectively by phenylalanine. These results show that the portions sensitive to different negative modifiers have individual properties.

A feature of these experiments was that there was no gain of non-inhibited activity. This means that either the active sites alone were lost, or that both active and inhibitor sites were lost together.

DISCUSSION

As explained in the introduction, DAHP synthase has been treated as a physiological unit and a potential physical unit of organisation. A number of tentative conclusions have been reached. With respect to substrates the kinetic treatment assumes that the active sites regulated by different kinds of inhibitor molecules have the same or very similar catalytic characteristics in the absence of these modifiers. The data is consistent with this hypothesis but is not unequivocal. Results suggest that for each effective catalytic unit there are a minimum of two sites for each substrate with strong homomolecular cooperation between them so that the substrates are also activators (positive modifiers).

A similar deduction can be made for the allosteric inhibitors phenylalanine, tyrosine and tryptophan, except that they are negative modifiers. In the example of phenylalanine and tyrosine the data best fits this conclusion when the total activity inhibited by phenylalanine and tyrosine is considered as a unit. There is evidence for synergism (heteromolecular cooperation) between phenylalanine and tyrosine which is consistent with a physical association between the active sites controlled by these allosteric ligands. In the example of tryptophan there is no evidence that the related active sites are associated with those controlled by the other negative modifiers. So

far it has not been possible to destroy the various negative modifier sites without also destroying the substrate-positive modifier sites.

Non-saturating levels of phenylalanine and tyrosine have little or no effect on $[S]_{0.5(V)}$ but lower m and v_{max}. It is concluded that inhibition is achieved by lowering inter-site cooperation and catalytic activity rather than the binding of substrate molecules.

In general terms the DAHP synthase of wild type *N. crassa* 74A has active sites of at least three kinds; those inhibited by phenylalanine, those inhibited by tyrosine and those specifically inhibited by tryptophan. It may be no accident that the ratio between them is about 44:44:10 in most extracts, thus corresponding to the approximate requirements for the aromatic amino acids. In all experiments with crude

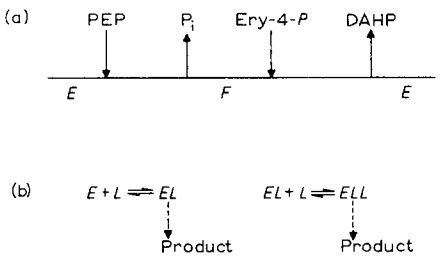


Fig. 12. A simplified mechanism for DAHP synthase. a, This illustrates the ping-pong nature of the reaction. PEP is thought to add before Ery-4-*P* and the overall reaction is physiologically irreversible. With purified preparations it should be possible to study the part reaction leading to P_i formation. b, It is postulated that all the ligands bind with the enzyme at a minimum of two sites with strong inter-site cooperation. In the example of either substrate (with the other saturating) the *ES* and *ESS* forms both yield the product DAHP. Inhibiting ligands are considered to bind with the enzyme reducing catalytic activity and driving the enzyme substrate interactions in the direction $ESS \rightarrow ES$, that is opposing substrate cooperation.

extracts, inhibition with high concns. (10^{-3} M of each) of the three negative modifiers approaches, but does not reach, 100%. A small residue (about 3%) of noninhibited activity remains. This may be related to the need to synthesise the vitamin 4-aminobenzoate.

It is likely that as for the phenylalanine-sensitive isoenzyme of *E. coli* the mechanism of the DAHP synthase reaction is ping-pong[23] (Fig. 12) but modified by the cooperative interactions already discussed. The sequential nature of the ping-pong mechanism and the protection by PEP (but not Ery-4-*P*) of the enzyme against heat denaturation leads to the conclusion that PEP is the first reactant. Thus, PEP can be regarded as the initiator of aromatic biosynthesis. The improved velocity characteristic of the non-cooperative behaviour of PEP at low concentration may be of regulatory significance in quickly achieving the physiological range when PEP is available for aromatic biosynthesis. Since Ery-4-*P* binds as the second reactant it will be spared in the absence of PEP.

In that the allosteric inhibitors are the aromatic amino acids, the DAHP synthase of *N. crassa* resembles that of *E. coli*[1,16,24–26] and *S. cerevisiae*[1,27–30] rather than *B. subtilis*[1,31]. However, the Neurospora enzyme differs in that Co^{2+} is a cofactor, ligand–enzyme interactions are cooperative and inhibition by tryptophan can readily be demonstrated. With regard to the cooperative kinetics of substrates, inspection of

the original paper describing the *E. coli* DAHP synthase[32] indicates that it was probably inappropriate to draw straight lines through the data of the double reciprocal plots. If the point at lowest dilution is ignored, curves, concave upwards, can be drawn. Similarly, the *v versus S* plots clearly are sigmoid. Other kinetic measurements[23,24] with *E. coli* preparations used partly purified preparations of the phenylalanine- and tyrosine-inhibited isoenzymes and properties may have changed accordingly.

For *E. coli*, repression of the isoenzymes is an important factor[1,33], in *N. crassa* and *S. cerevisiae* significant repression below normal wild-type levels has not been detected[1,27–29] *N. crassa* differs from *S. cerevisiae* in that the DAPH synthase of the latter has not been shown to be tryptophan inhibited[28].

In follow-up to the work reported in this paper it has been possible to show that DAHP synthase isoenzymes exist[8]. Molecular sieving separates isoenzymes distinguished on the basis of estimated molecular weight and sensitivity to inhibition. The recognition of the phenylalanine- and tyrosine-sensitive isoenzymes depends on the presence of PEP and Co^{2+}. The use of PEP and Co^{2+} as stabilising agents derived from the work in this paper. Co^{2+} is probably concerned with various protein–protein interactions. These analyses and the relation between the various isoenzymes and three separate structural genes of DAHP synthase will be presented elsewhere[7–9]. With regard to the present paper these later studies confirm the physiological unity of DAHP synthase and illustrate the necessity for the present work as a preliminary to interpretation of the data with "purified" preparations.

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