THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 241, No. 1, Issue of July 25, pp. 3373-3380, 1966
Printed in U.S.A.

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

II. THE ENZYMOLOGY OF FEEDBACK INHIBITION OF 3-DEOXY-D-*ARABINO*-HEPTULOSONATE 7-PHOSPHATE SYNTHETASE*

(Received for publication, February 3, 1966)

ROY A. JENSEN‡ AND EUGENE W. NESTER

From the Departments of Microbiology and Genetics, School of Medicine, University of Washington, Seattle, Washington 98105

SUMMARY

Enzymological studies of the action of the inhibitors, chorismate and prephenate, upon purified preparations of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthetase in *Bacillus subtilus* are described for an allosteric system of feedback inhibition in aromatic amino acid biosynthesis.

1. The K_i values for chorismate and prephenate are 2.8×10^{-4} M and 4.0×10^{-5} M, respectively.

2. The following evidence is consistent with the absence of intersite interactions between substrate-binding sites: (*a*) the observation of first order kinetics in substrate saturation curves; (*b*) strictly noncompetitive inhibition; and (*c*) the determination of an interaction coefficient, $n = 1.0$, with the Hill system of coordinates.

3. Cooperative inhibitions were observed between inhibitor-binding sites. Plots of kinetic data take the form of sigmoid inhibition curves. The interaction coefficient, n', is 1.5 at inhibitor concentrations causing more than 35% inhibition of enzyme activity. Lower concentrations yield a value of 2.8 for n'.

4. Inhibition of DAHP synthetase by chorismate and prephenate is potentiated at low temperatures. This effect of temperature is correlated with the alteration of the overall dissociation constant, K. The cooperative interactions between substrate-binding sites are independent of temperature as judged by measurements of interaction coefficients at different temperatures.

5. Monomer-polymer protein interactions probably do not play a major role in the feedback inhibition of DAHP synthetase by intermediary metabolites in aromatic amino acid synthesis since the sedimentation pattern of the enzyme is unchanged in the presence of inhibitors.

6. DAHP synthetase is activated by *p*-chloromercuribenzoate and iodoacetate and these compounds potentiate the inhibition of the enzyme. In contrast, 2-mercaptoethanol inhibits DAHP synthetase and partially desensitizes the enzyme to inhibitor effects.

7. DAHP synthetase is partially desensitized with mercuric acetate. Heat treatment and exposure to urea have no effect upon inhibition of remaining enzyme activity. Total desensitization is never observed. Neither the feedback effectors nor the aromatic amino acids protected the enzyme from a number of inactivating agents.

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

‡ Present address, Department of Biology, State University of New York at Buffalo, Buffalo, New York.

Recently the importance of a ubiquitous class of enzyme proteins known as "regulatory enzymes" (1) has been recognized. These studies have emphasized the molecular mechanisms responsible for adjustments in catalytic activity that may occur in the presence of certain low molecular weight metabolites. Such studies have been a natural extension of the discovery that multienzyme sequences of biochemical reactions could be self-regulated (feedback control) by the interactions of terminal metabolites (effectors) and the first enzyme of the sequence (2). Early investigations of the enzymology of feedback-inhibited enzymes (3–5) resolved the apparent paradox that metabolites that were grossly dissimilar to substrate molecules in stereochemical configuration nevertheless behaved as highly specific inhibitors. The eventual appreciation of the existence of spatially distinct attachment sites on the enzyme surface for both substrate and effector molecules has led to the general adoption of the adjective "allosteric" (6) to describe indirect interactions between substrate and effector molecules. The molecular basis for the alteration of catalytic properties is thought to depend upon the quaternary (subunit) structure of enzyme molecules (7). Presumably the binding of allosteric effectors influences enzyme catalysis by promoting conformational or other changes in quaternary associations of protein subunits.

Regulatory enzymes have been grouped into two classes (7, 8) on the basis of the influence of the effector molecule upon the classical kinetic parameters that characterize enzyme activity, the affinity constant (K_m) and molecular activity (V_{max}). Thus,

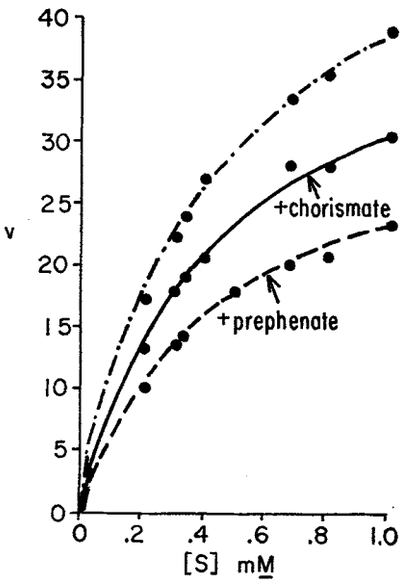

FIG. 1. Substrate saturation curve. Variable substrate is P-enolpyruvate. Erythrose-4-P concentration was 2.0 mM. *Unlabeled curve*, reaction velocity determined in the absence of inhibitors. Inhibitor concentrations: chorismic acid, 0.09 mM; prephenic acid, 0.03 mM. The initial reaction velocity, v, is expressed as millimicromoles of DAHP formed per min per ml of reaction mixture. An extract from strain WB 698b was prepared by sonic disruption and purified 11-fold by the use of Procedure 1 (9). Curves of similar shape were obtained when erythrose-4-P was used as the variable substrate with P-enolpyruvate concentration constant at 2.0 mM. Reaction time, 10 min at 37°.

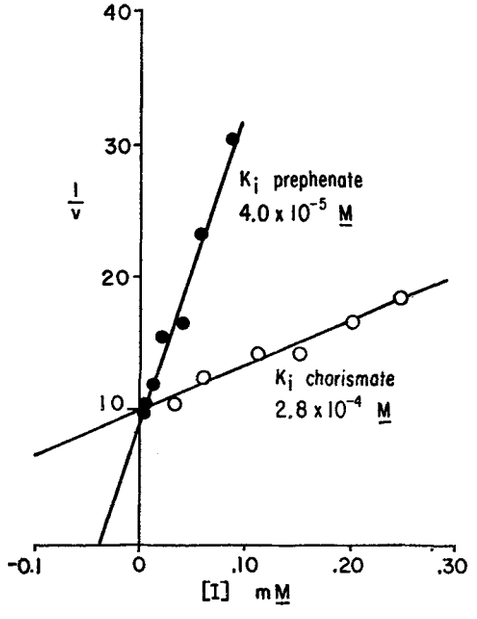

FIG. 2. Determination of inhibitor constants, K_i, by Dixon plot of reciprocal of reaction velocity, $1/v$, against inhibitor concentration $[I]$. Each *point* is an average of three determinations. An extract from strain SB 167, purified 45-fold by Procedure 3 (9), was used. The initial reaction velocity, v, is expressed as micromoles of DAHP formed in 1 min per mg of protein.

enzymes that possess an altered apparent K_m in the presence of effector belong to the K system, whereas those enzymes the effectors for which alter the apparent V_{max} belong to the V system. 3-Deoxy-D-*arabino*-heptulosonate 7-phosphate synthetase in *Bacillus subtilis* is a single enzyme protein (9) which is noncompetitively inhibited by prephenate and chorismate with respect to both of its substrates, erythrose-4-P and P-enolpyruvate (10). Therefore, DAHP[1] synthetase belongs to the V system category of enzymes. The relatively few allosteric proteins which have been studied in sufficient detail to classify them in the V system include adenosine triphosphate-5-phosphoribosylpyrophosphate pyrophosphorylase of *Salmonella typhimurium* (5), homoserine dehydrogenase of *Escherichia coli* (11), and the lysine-sensitive aspartokinase (aspartokinase II) of *E. coli* (12). The relatively good stability (9) of DAHP synthetase permitted its comparison with other V system enzymes.

EXPERIMENTAL PROCEDURE

The preparation of chorismate and prephenate, sources of biochemical compounds, procedure for assaying the activity of DAHP synthetase, characteristics of the enzyme, and methods used for its purification have been described (9, 10). Other experimental details are given in the legends. Most of the inhibition data in this communication were obtained by the use of prephenate. Similar experiments with chorismate as the inhibiting compound gave comparable results without exception.

When zone centrifugation was carried out with inhibitor-treated extracts, the sucrose gradient contained phosphate buffer with saturating concentrations of inhibitor (4.0 mg per ml of chorismate; 1.0 mg per ml of prephenate). The procedure and use of internal standards were previously described (9).

RESULTS

Kinetic Analysis of Inhibition

Cooperative Inhibition—The form of the substrate saturation curve for DAHP synthetase conforms with Michaelis-Menten kinetics (Fig. 1). Even in the presence of inhibitor, there is no indication of the sigmoid kinetics which implicates cooperative binding of substrate molecules. The kinetic parameters of V_{max} and K_m for DAHP synthetase in *B. subtilis* have been reported (9); the Michaelis constants are 1.1×10^{-3} M for erythrose-4-P and 4.8×10^{-4} M for P-enolpyruvate. The graphical method of Dixon (13) has been used in Fig. 2 for the determination of inhibitor constants (K_i). The K_i values for chorismate and prephenate are 2.8×10^{-4} M and 4.0×10^{-5} M, respectively. The inhibition of DAHP synthetase by chorismate and prephenate is freely reversible, specific, and noncompetitive. Other available intermediary metabolites do not inhibit the activity of DAHP synthetase (10). We have recently found that the immediate bio-synthetic precursor of chorismate, 3-enolpyruvylshikimic acid 5-phosphate (14) (generously supplied by Dr. David Sprinson) also is not inhibitory.

Fig. 3 shows the sigmoid form of inhibition curves obtained with DAHP synthetase from two different strains of *B. subtilis*, strains 168 and 23. According to Monod *et al.* (1) such sigmoid curves of inhibition can be ascribed to cooperative interaction between inhibitor sites on the enzyme molecule. While th

[1] The abbreviations used are: DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; CMB, *p*-chloromercuribenzoate.

general form of the inhibition curves is similar, there is a significant quantitative difference in the sensitivity of the two enzymes to the inhibitors; the enzyme activity of strain 23 is inhibited 50% at 0.0175 mM prephenate compared to a required prephenate concentration of 0.0300 mM to achieve equal inhibition in strain 168.[2]

Influence of Temperature—The ability of enzyme and inhibitor to form an inactive complex is remarkably temperature-dependent. Relative activity is plotted in Fig. 4 as a linear function of incubation temperature at two concentrations of prephenate. A precipitous decrease in the inhibitory action of prephenate upon enzyme activity accompanies the increase of reaction temperature. Thus, inhibitor concentrations which cause only marginal inhibition at 46° inhibit enzyme activity about 80% at 20°. The temperature range (Fig. 4) is well within the limits of thermal stability for DAHP synthetase (9). Saturating concentrations of inhibitor at 37° produce maximal levels of inhibition of about 83%; however, such residual enzyme activities at saturating concentrations of inhibitor ($v_{i\,(sat)}$) vary as a function of temperature and the value of $v_{i\,(sat)}$ decreases at lower temperatures. At high concentrations of inhibitor, the curves approach 100% inhibition (*i.e.* $v_{i\,(sat)} = 0$) asymptotically as temperature is decreased. The temperature-dependent association of inhibitor and enzyme is freely reversible; a given concentration of inhibitor decreases the reaction velocity to a level characteristic of the assay temperature, independently of prior incubation of enzyme and inhibitor at higher or lower temperatures. The effectiveness of inhibitors is also independent of the order of addition of substrates and inhibitors to enzyme preparations at various temperatures. Presumably, this temperature effect could be a consequence of a change in the effective concentration of inhibitor owing to (*a*) an increase in the over-all association constant with decreasing temperatures or (*b*), less likely, a temperature-dependent alteration of two or more molecular species of inhibitor. The sigmoid inhibition curves obtained at 19, 37, and 44° are shown in Fig. 5. It is clear that the range of inhibitor concentrations spanning negligible to maximal inhibitions decreases substantially at lower temperatures, and varies over a relatively broad range at higher temperatures. In normal plotting procedures compression of the scale of inhibitor concentration would tend to obscure the sigmoid shape of the curve at 44°. For example, if the inhibition data obtained at 19 and 44° are normalized to the inhibitor concentrations yielding about 80% inhibition (Fig. 6), the inhibitor concentration scale at 44° is compressed about 20-fold. The sigmoid shape of the curve obtained at 19° is more obvious than the one determined at 44° in Fig. 6. Further compression of the inhibitor concentration axis at 44° yields a curve approximating the form of a rectangular hyperbola, a result which might easily be interpreted as noncooperative kinetics of inhibition. Atkinson, Hathaway, and Smith (16) have discussed analogous results in which an apparent change in the kinetic order of yeast phosphofructokinase in substrate is attributed to unsatisfactory measurements of reaction velocity at low substrate concentrations. It is clear from Fig. 5 that the apparent affinity of the enzyme for inhibitor varies over a very large range of inhibitor concentration, depending upon temperature. A comparison of Fig. 5 and Fig. 6 illustrates that conventional plots of inhibition data obtained at higher temperatures tend to minimize experimental points taken at low inhibitor concentrations. The conclusion that temperature does not influence interactions among inhibitor sites is best substantiated by the plot used in Fig. 7.

Interaction Between Inhibitor Binding Sites—If the standard Michaelis treatment is applied to the case of an enzyme with n

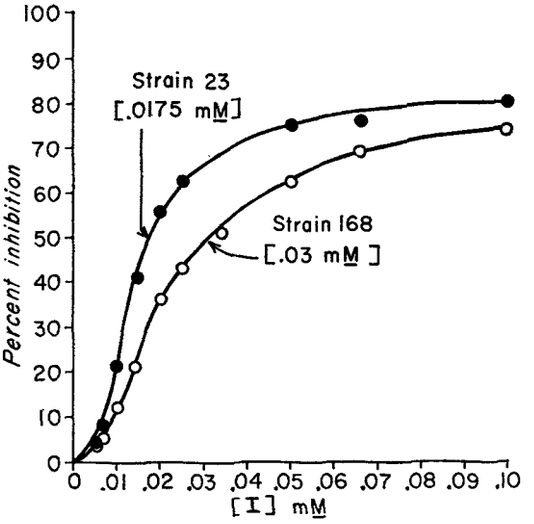

FIG. 3. Inhibition curves in two *B. subtilis* strains. *Arrows* indicate concentration of prephenate [*I*] inhibiting the activity of DAHP synthetase 50%. A similar experiment with chorismic acid as inhibitor produced 50% inhibition in strains 168 and 23 at chorismate concentrations of 0.24 mM and 0.40 mM, respectively. Crude extracts were used: strain 168 specific activity, 21.0 mμmoles per min per mg of protein; strain 23 specific activity, 37.5 mμmoles per min per mg of protein. Reaction time, 10 min at 37°. Percentage inhibition equals $(v_0 - v_i)/v_0$.

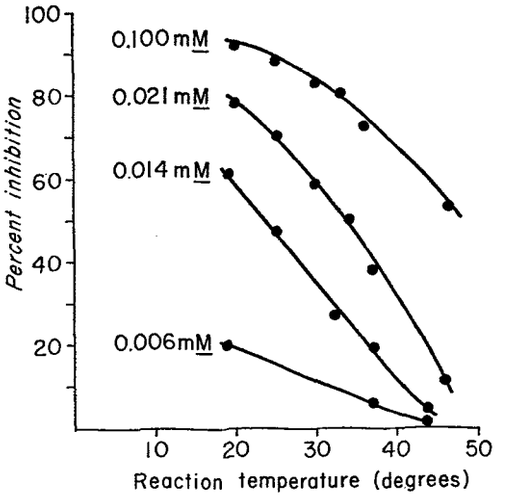

FIG. 4. Effect of incubation temperature upon inhibition of DAHP synthetase activity by prephenate. The extract is the same as that described under Fig. 1. Reaction time, 15 min. Temperatures were maintained within a variance of 0.5°. Each *experimental point* is an average of four determinations in two experiments. Percentage inhibition equals $(v_0 - v_i)/v_0$.

[2] The reader is referred to the results of Nester and Jensen (15) where analogous differences between these strains are described in the cases of anthranilate synthetase and prephenate dehydratase.

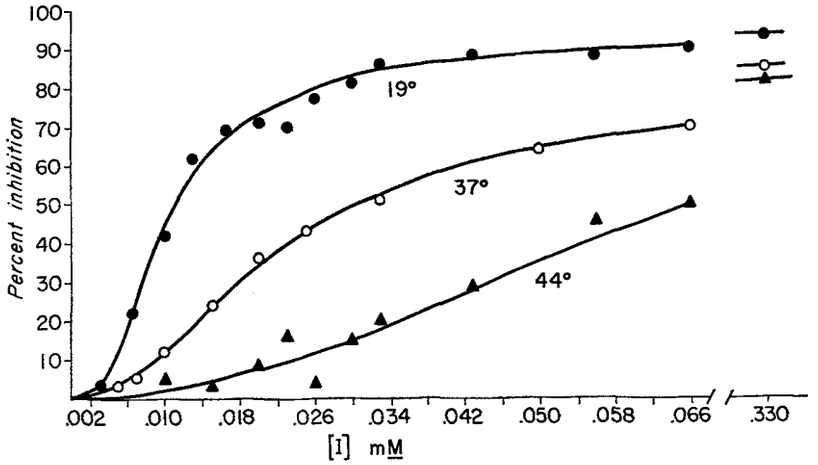

FIG. 5. Inhibition curves at various temperatures in the presence of prephenate. The reaction velocities at 0.33 mM prephenate are very close to the values used for $v_{i\,(sat)}$. The extract was made from SB 167, purified 11-fold by Procedure 1 (9). Percentage inhibition equals $(v_0 - v_i)/v_0$. Reaction times: 10, 15, and 20 min at 44, 37, and 19°, respectively.

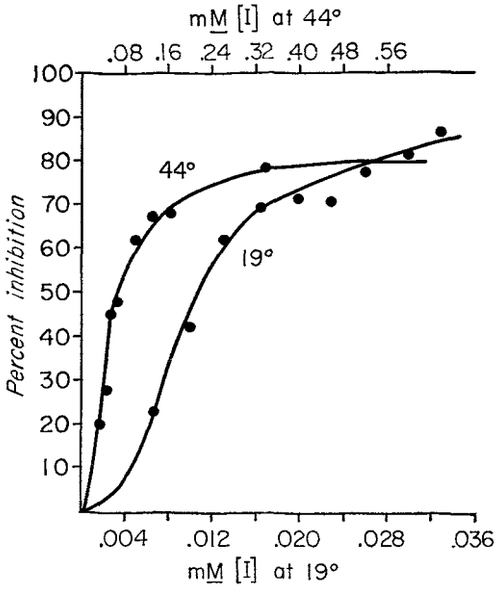

FIG. 6. The apparent effect of temperature upon sigmoid shape of inhibition curve. The data of Fig. 5 are replotted by normalizing to prephenate concentrations causing 80% inhibition at each temperature. The *abscissa scale* at 44° is compressed 20-fold with respect to the *abscissa scale* at 19°.

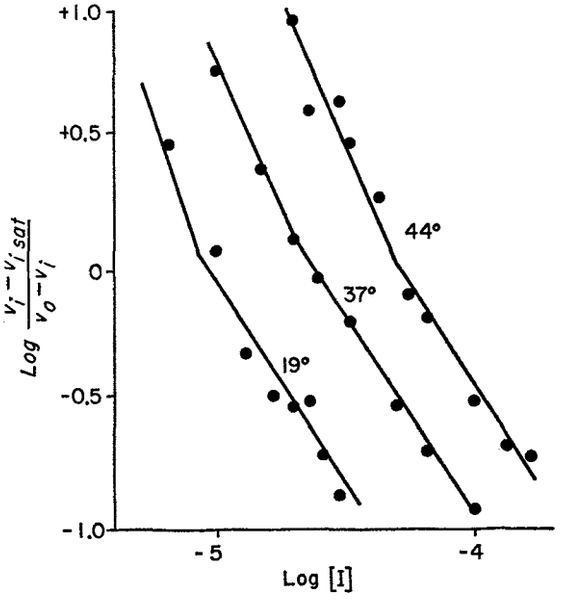

FIG. 7. Determination of interaction coefficient, n', between inhibitor binding sites. The data are plotted from that of Fig. 5. The values used for $v_{i\,(sat)}$ at 44, 37, and 19° were 19, 17, and 8% of v_0, respectively. The break in slope for each curve is drawn at points corresponding to 35% inhibition. The negative slope, n', equals 2.8 at low inhibitor concentrations and 1.5 at higher inhibitor concentrations.

mutually interacting sites, then

$$K = \frac{(E)(S^n)}{(E \cdot S_n)} \qquad (1)$$

where E = free and active enzyme, n = apparent number of substrate-binding sites per enzyme molecule, $E \cdot S_n$ = complex of enzyme and n substrate molecules, and K = apparent over-all dissociation constant.

The application of the Michaelis-Menten treatment to the derivation of Equation 2, otherwise known as the empirical Hill equation (17)

$$\log \frac{v}{V_{max} - v} = n \log (S) - \log K \qquad (2)$$

has been given by Atkinson *et al.* (18). A plot of

$$\log \frac{v}{V_{max} - v}$$

against log (S) gives a line of slope n, otherwise termed the interaction coefficient (19). Thus, n is a measure of cooperative interactions between substrate molecules. If one makes the analogous derivation for the case in which an enzyme molecule possesses n interacting binding sites for inhibitor ligands and where the following equilibrium reaction is assumed,

$$E + I_n \rightleftharpoons (E \cdot I_n)$$

then rearrangement of the identity for K, the apparent product of n dissociation constants, gives

$$\log \frac{v_i}{v_0 - v_i} = \log K - n' \log (I) \quad (4)$$

where v_i = reaction velocity in presence of inhibitor and v_0 = reaction velocity in absence of inhibitor.

If one uses association constants instead of dissociation constants for K (20), then

$$\log \frac{v_0 - v_i}{v_i} = \log K + n' \log (I) \quad (5)$$

and n' in the appropriate plot will be expressed as the value of the positive slope rather than the negative value of the slope obtained from the use of Equation 4. The designations n, n', and n'' refer to interaction coefficients measured between substrates, inhibitors, and activators, respectively (8). The numerical value of the interaction coefficient depends both on the number of interacting sites and the strength of interactions between them. This coefficient is independent of the number of sites and equals 1.0 in the absence of cooperative binding. If the interactions are strong, the coefficient approaches a limiting integral value equal to the number of sites. The application of Equation 4 (or 5) for the estimation of cooperative inhibitions has been made (19, 20) in systems where inhibition approaches 100%. However, when the inhibition observed at saturating concentrations of inhibitors is less than 100%, it can be calculated that since

$$\frac{K}{(I)^n} = \frac{(E)}{(E \cdot I_n)} \quad (6)$$

then

$$\log \frac{v_i - v_{i(\text{sat})}}{v_0 - v_i} = \log K - n' \log (I) \quad (7)$$

where $v_{i(\text{sat})}$ is the reaction velocity at saturating concentrations of inhibitor. In the case of DAHP synthetase, $v_{i(\text{sat})}$ at 37° equals 17% v_0.

If the Hill system of coordinates is applied to kinetic measurements of DAHP synthetase activity by plotting $\log v/(V_{\max} - v)$ against $\log (S)$ where S is either P-enolpyruvate or erythrose-4-P, one obtains a straight line of positive slope, n = 1.0. This is interpreted to mean that substrate binding sites are independent, particularly since the further addition of inhibitors does not alter the shape of the substrate saturation curve. This is, of course, consistent with the strictly noncompetitive kinetics of inhibition observed in the presence of chorismate and prephenate.

If Equation 7 is applied to obtain the coordinates of the plot used in Fig. 7, the negative slope n' exceeds a value of 1.0; therefore, the inhibitory effectors exhibit cooperative interactions. The plots in Fig. 7 are straight, essentially parallel lines. They are characterized by a rather sharp transition in slope giving a greater value of n' at low inhibitor concentrations. This transition occurs at inhibitor concentrations corresponding to about 35% inhibition at each temperature (compare with Fig. 6). Below inhibitor concentrations giving 35% inhibition (0.0084 mM, 0.0200 mM, and 0.0500 mM at 19, 37, and 44°, respectively) n' equals 2.8; above these inhibitor concentrations n' equals 1.5. Perfectly straight lines have been obtained in such log-log plots over the entire range of effector concentration (16, 20) and presumably characterize particularly strong cooperative interactions. However, curves which do not maintain a constant slope at all effector concentrations are probably more typical (8, 21, 22). Clearly, the curves corresponding to each temperature in Fig. 7 are parallel and, therefore, represent the same degree of cooperativeness between inhibitor molecules. These curves differ in their displacement to the left at lower temperatures. Since the intercept on the ordinate is equal to the value of K (Equation 7), the over-all dissociation constant increases at higher temperatures. The cooperative interactions between inhibitor molecules are entirely independent of temperature. This measure of the interaction coefficient sets the minimum number of inhibitor sites per enzyme molecule at three.

When the inhibition data are plotted (Fig. 8) as the ratio of inhibitable activity in the absence of inhibitor to inhibitable activity in the presence of inhibitor, $(v_0 - v_{i(\text{sat})})/(v_i - v_{i(\text{sat})})$, against inhibitor concentration, the resulting curves bend away from the coordinate of inhibitor concentration at low inhibitor concentrations. This is consistent with the interpretation that 1 enzyme molecule interacts with more than 1 inhibitor molecule, a necessary prerequisite for cooperative interactions of inhibitor molecules. A straight line relationship in such a plot would be expected for the case in which one inhibitor molecule binds to one enzyme molecule.

Conformational Alterations of DAHP Synthetase

Relationship of Inhibition and Molecular Weight of Enzyme—Since the binding of effector to enzyme has sometimes been observed to control catalytic activity by influencing the equilibrium association-dissociation of subunit proteins (23), the possible change of the molecular weight of DAHP synthetase in the presence of inhibitors was investigated. An extract from a derepressed strain, purified 53-fold by Procedure 3 (9), was cleaved into three portions. The sedimentation of enzyme centrifuged in sucrose density gradients containing either prephenate or chorismate (9) was compared to the sedimentation of enzyme activity

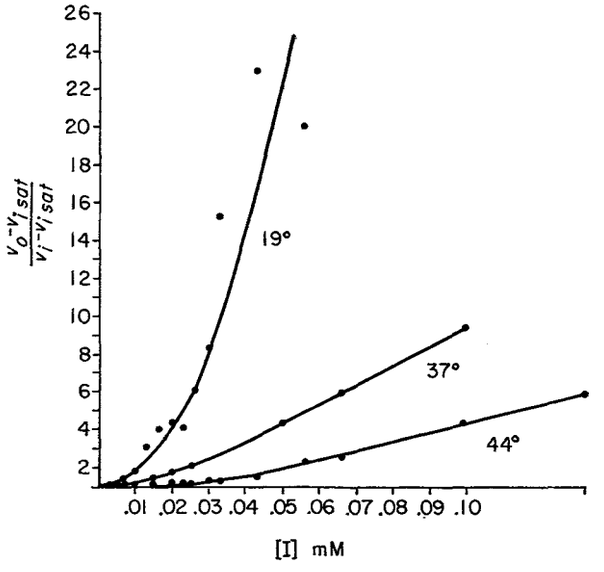

FIG. 8. Kinetic analysis indicating that 1 enzyme molecule binds more than 1 inhibitor molecule. The data are taken from Fig. 5.

in the absence of the inhibitor. No detectable difference in sedimentation velocity could be discerned and all three gradients had peak enzyme activity in the same fraction.

Certain sulfhydryl reagents influence both the catalytic site and the inhibitor site of DAHP synthetase, a result that is probably best explained by conformational changes induced in the enzyme protein. DAHP synthetase not only is resistant to inhibition by *p*-chloromercuribenzoate but is stimulated at least 2-fold by optimal concentrations as shown in Fig. 9. Comparable stimulation of enzyme activity was observed in both crude extracts and partially purified preparations. The activating effect of CMB is greatest at about 10^{-5} M; both higher and lower concentrations of CMB produce less stimulation. The enzyme is stable to 10^{-5} M CMB for at least 6 hours, but longer exposures result in total inactivation. Other organic mercurials have not been tested yet. However, a nonmercurial sulfhydryl reagent, iodoacetate, also stimulates enzyme activity to about the same extent as CMB at similar concentrations. On the other hand, mercuric acetate did not stimulate activity at any concentration and was quite inhibitory (see Table II). Preincubation of the enzyme with CMB up to several hours did not influence the magnitude of the activation effect. This stimulatory effect of certain sulfhydryl reagents has not been tested as a function of pH or ionic strength. In contrast, the enzyme activity is inhibited by 2-mercaptoethanol, a reagent which acts to bring the enzyme reaction to completion in *E. coli*. Srinivasan and Sprinson (24) have reported that 2-mercaptoethanol concentrations of 2×10^{-3} M inhibit the assay system about 27 to 30%. The inhibition data given in Fig. 9 have not been corrected for the inhibition by 2-mercaptoethanol of the assay system for DAHP. However, the inhibition shown in Fig. 9 is substantially greater than this. For example, an inhibition of 30% occurs at a concentration of about 10^{-5} M, a concentration which does not inhibit the assay system. That the stimulation of activity by CMB and the inhibition by 2-mercaptoethanol may be expressions of the same basic phenomenon is supported by the abolition of these effects by the simultaneous addition of appropriate concentrations of these reagents.

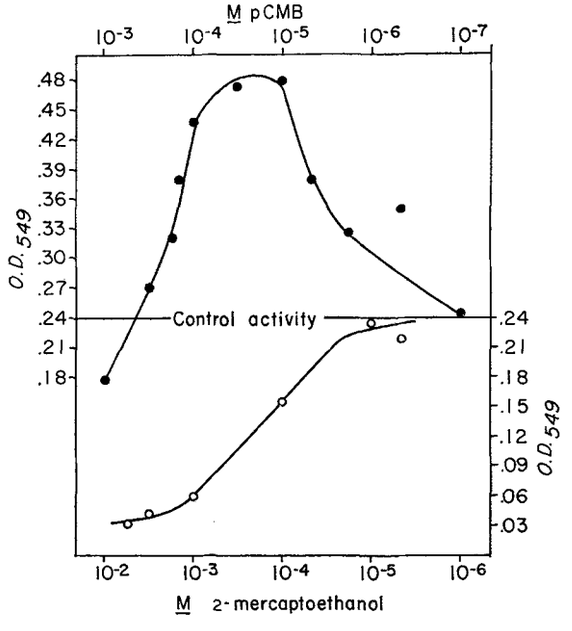

FIG. 9. Influence of *p*-chloromercuribenzoate (*pCMB*) and 2-mercaptoethanol upon activity of DAHP synthetase. The extract was made from strain 698b, purified 10-fold by Procedure 1 (9). Components of enzyme reaction were adjusted to a pH of 6.5. CMB and 2-mercaptoethanol were added to the enzyme preparation 30 min before the addition of substrates. Enzyme activity is expressed as optical density at 549 mμ per 10 min of reaction time at 37°. Inhibition by 2-mercaptoethanol is uncorrected for inhibition of the assay system (see text). Control activity was determined in the absence of CMB and 2-mercaptoethanol.

TABLE I

Agents which sensitize or desensitize DAHP synthetase to inhibitor effects

The extract was the same described under Fig. 9. Iodoacetate, CMB, or 2-mercaptoethanol was added to the enzyme preparation 20 min before the addition of substrates. Prephenate was added to one tube in each set 5 min before the addition of substrates. The conditions represented by the first and second columns of each set differ only in the presence or absence of prephenate. Percentage inhibition by prephenate is calculated as the ratio $(v_0 - v_i)/v_0$ where v_0 is the control reaction velocity after activation by CMB or iodoacetate or after inhibition by 2-mercaptoethanol and v_i is the reaction velocity of an identical reaction mixture also containing prephenate. Reaction time, 10 min at 37°.

Concentration of agent	CMB		Iodoacetate		2-Mercaptoethanol	
	Activation	Inhibition by prephenate[a]	Activation	Inhibition by prephenate[a]	Activation	Inhibition by prephenate[b]
M	%	%	%	%	%	%
None		41		39		70
3.0×10^{-3}	10	40	4	41	82	44
1.6×10^{-3}	25	44	21	45	76	37
1.0×10^{-4}	83	50	84	54	33	51
3.0×10^{-5}	95	67	105	71	16	68
1.0×10^{-5}	101	66	111	65	3	67
1.0×10^{-6}	20	48	24	44		

[a] Prephenate concentration, 0.022 mM.

[b] Prephenate concentration, 0.070 mM.

Integrity of Inhibitor Site—Gerhardt and Schachman (25) found that the catalytic subunit of aspartate transcarbamylase of *E. coli* was more active when it was dissociated from the regulatory subunit. The CMB stimulation of enzyme activity is not a consequence of a similar desensitization of the enzyme to inhibitor effects since the CMB-activated DAHP synthetase is actually somewhat more sensitive to inhibition than control preparations. Table I shows the correlation of increase in the sensitivity of DAHP synthetase with the activation of the enzyme. There is an analogous loss in sensitivity to inhibitor in the presence of 2-mercaptoethanol. Apparently the conformational changes induced by sulfhydryl reagents either (*a*) enhance catalytic activity in parallel with an increased binding of the inhibitor at the inhibitor site (*e.g.* CMB) or (*b*) decrease both catalytic activity and the affinity of the inhibitor site for inhibitor (*e.g.* 2-mercaptoethanol).[3]

[3] In one purification procedure an ammonium sulfate fraction (50 to 55% saturation), which was stored frozen at −20° for 1 week spontaneously dissociated to give another molecular weight species of about 60,000 molecular weight judged by its elution from a Sephadex G-100 gel column. This preparation behaved like the

TABLE II

Effect of various inactivating agents upon sensitivity to inhibition

Urea was adjusted to pH 6.5 and incubated with the enzyme at 0° for 25 min before the addition of substrates to the reaction mixture. The extract was prepared from SB 167, purified 13-fold by Procedure 1 (9). Reaction time, 10 min at 37°.

Inactivation conditions	Per cent of initial activity	Per cent inhibition of initial activity
Concentration of mercuric acetate[a]		
None	100	65
1 μM	78	61
2 μM	78	62
3 μM	39	47
4 μM	13	4
6 μM	2	0
8 μM	0	
Time of heat treatment (66°)[b]		
None	100	80
2 min	95	75
3 min	88	79
4 min	68	80
5 min	50	79
6 min	37	81
Concentration of urea[b]		
None	100	78
0.3 M	92	81
0.5 M	85	75
1.0 M	73	83
1.5 M	60	79
2.5 M	48	79

[a] Prephenate concentration, 0.050 mM.

[b] Prephenate concentration, 0.250 mM.

Relative Stability of Catalytic and Effector Sites—Many physical treatments have been described which maintain the integrity of the catalytic site of enzymes but selectively disrupt the effector site. Such densensitization procedures include the exposure of enzymes to heat and the use of mercury-containing compounds and urea. Spontaneous desensitization may occur following aging, dialysis, freezing, or purification (3–5, 11, 12, 21). The previously described effects of CMB, iodoacetate, and 2-mercaptoethanol result in only partial desensitizations (or potentiations) to inhibition. In Table II the sensitivity of DAHP synthetase to inhibition is measured after treatment of the enzyme with mercuric acetate. Concentrations of mercuric acetate which cause negligible inactivation of the enzyme likewise have little effect upon its susceptibility to inhibitor ligands. However, at higher concentrations, as relatively more inactivation occurs, less of the residual activity is inhibited. It will be recalled that this effect of an inorganic mercurial is opposite to the effects of the organic mercurial, CMB. Not even partial desensitization was observed following heat treatment or exposure to urea (Table II). In these cases the enzymic activity inhibited was constant, even where substantial inactivation of the enzyme occurred. Enzyme preparations as old as 6 months (aged extracts) were never observed to have altered sensitivities to feedback effectors. The substitution of Tris buffer for the usual phosphate buffer did not influence the outcome of the above attempts to desensitize the enzyme.

CMB-activated enzyme is that (*a*) the specific activity increased almost 2-fold over that of the original preparation, (*b*) it was more sensitive to inhibitor than the control extract, and (*c*) it was not activated by CMB but was still inhibited by 2-mercaptoethanol. It was also rather unstable, losing activity after 1 week. So far suitable conditions have not been found to reproduce this change in the molecular weight and properties of the enzyme. The possibility that CMB-activated DAHP synthetase might have a decreased molecular weight has not yet been checked.

TABLE III

Stability of DAHP synthetase in presence of small molecules

Prephenate and chorismate at these concentrations were slightly inhibitory (11% and 5%, respectively); the figures given in the table were corrected by comparison with untreated control extracts. The extract was the same as the one described in Table II. Reaction time, 10 min at 37°.

Conditions	Activity remaining after treatment		
	66°, 5 min	Urea, 2.5 M, 25 min	Mercuric acetate, 3.0×10^{-6} M
	%	%	%
No additions	44	52	33
+ Prephenate, 10^{-5} M	49	47	33
+ Chorismate, 5×10^{-5} M	44	54	37
+ Aromatic amino acids,[a] 2.5×10^{-4} M	45	57	31

[a] Aromatic amino acids constitute the indicated concentrations of each of the L-isomers of tyrosine, phenylalanine, and tryptophan.

Allosteric effectors have frequently been reported to protect enzymes from various inactivating agents (6, 12, 26, 27). This behavior is consistent with the induction of conformational changes by effector molecules. The presence of chorismate or prephenate does not appear to protect the enzyme from thermal inactivation at the concentrations used in Table III. Likewise, aromatic amino acids at concentrations of 3.0×10^{-4} M do not protect the enzyme. The decreased stability of partially purified DAHP synthetase (9) to prolonged dialysis (>16 hours) is not influenced by the presence of chorismate or prephenate during dialysis. However, enzyme stabilization by chorismate and prephenate may be found under other conditions *in vitro*.[4]

DISCUSSION

Regulatory enzymes of the K system type (7, 11) have been thoroughly studied because of their potentially significant role in the distribution of common intermediates at metabolic branch points (28). The influence of effector molecules upon cooperative interactions between substrate molecules that do not follow Michaelis kinetics has been emphasized in the literature (16, 19, 29). An explanation of the complex mutual dependencies of various enzyme-bound reaction components has been made by Atkinson and Walton (30). In contrast, enzymes which can be classified in the V system have received less attention. The DAHP synthetase of *B. subtilis* represents the V system on the following grounds. (*a*) The substrate saturation curve can be fitted to an approximation of a rectangular hyperbola rather than the sigmoid curve characteristic of cooperative interactions between substrate molecules. The Hill plot yields an interaction coefficient of $n = 1$. Hence, no homotropic interactions

[4] Significant differences in the long term storage stability of crude extracts occur depending upon the nutritional history of the cultures from which various extracts were prepared.

occur between substrate molecules. (*b*) Homotropic interactions between inhibitor molecules occur as predicted by Monod *et al.* (7) for V systems. This cooperative effect is apparent in the sigmoid form of inhibition curves (Figs. 3, 5, and 6), by the graphical evaluation of the interaction coefficient where $n' > 1.0$ (Fig. 7), and the kinetic plot (Fig. 8) suggesting that 1 enzyme molecule interacts with more than 1 inhibitor molecule. (*c*) The inhibition is strictly noncompetitive as clearly seen in double reciprocal plots (10). Therefore, inhibition occurs through an alteration in molecular activity (V_{max}) rather than by changing the affinity for substrate (K_m).

Desensitization—One generalization often made about regulatory enzymes (based on relatively few well studied cases) is that the effector site is more fragile than the catalytic site. However, conditions favoring the selective destruction of the effector binding site have not been observed for some regulatory enzymes (12, 31). DAHP synthetase belongs to the latter category of enzymes, although partial desensitizations were observed with the use of mercuric acetate and with 2-mercaptoethanol. In addition, the inhibitors did not appear to protect the enzyme from the effects of a variety of inactivating agents. The inhibitor- and substrate-binding sites appear to have similar, if not mutually dependent stabilities.

Temperature Dependence of Inhibition—The variation in degree of inhibition as a function of temperature has not been studied in the case of most well known allosteric proteins. However, it is possible that the increased inhibition of enzyme activity at lower temperatures may be a common feature of regulatory enzymes. Similar temperature-dependent inhibition relationships have been obtained for ribose 5-phosphate-ATP pyrophosphorylase of *E. coli* (32) and for fructose 1,6-diphosphatase in rat liver (20). This phenomenon may be related to the different steady state concentrations of metabolites that may exist in metabolic pools at different temperatures. A decreased intracellular concentration of feedback inhibitor at lower temperatures of growth could act to maintain the same relative degree of enzyme inhibition in force at higher temperatures. Hence, the physiological significance of temperature modulation of the control system would be the adjustment of the control mechanism to fluctuations of internal inhibitor concentrations with temperature. The variation in the value of $v_{i(sat)}$ at different temperatures indicates that the residual activity observed at higher temperatures with saturating concentrations of inhibitor cannot be attributed to an inherently insensitive species of enzyme.

CMB Activation—The unusual activation of DAHP synthetase by CMB and iodoacetate and its inhibition by 2-mercaptoethanol are probably the consequence of a conformational change in the enzyme protein. The slight but significant increased sensitivity of CMB-activated enzyme and the decreased sensitivity of 2-mercaptoethanol-inhibited enzyme support this interpretation. The partial desensitization of DAHP synthetase with mercuric acetate also suggests the involvement of conformational changes. Several examples of stimulation of enzyme activity by CMB have been reported recently. Rogers *et al.* (33) concluded that CMB stimulation of enzyme activity was caused by the exposure of more active sites as a consequence of the dissociation of the enzyme into subunits. Perkins and Bertino (34) suggest that their results can be attributed to the production of a conformational change following treatment with CMB.

REFERENCES

1. Monod, J., Changeux, J. P., and Jacob, F., *J. Mol. Biol.*, **6**, 306 (1963).
2. Umbarger, H. E., *Science*, **145**, 674 (1964).
3. Changeux, J. P., *Cold Spring Harbor Symp. Quant. Biol.*, **26**, 313 (1961).
4. Gerhart, J. C., and Pardee, A. B., *J. Biol. Chem.*, **237**, 891 (1962).
5. Martin, R. G., *J. Biol. Chem.*, **238**, 257 (1963).
6. Monod, J., and Jacob, F., *Cold Spring Harbor Symp. Quant. Biol.*, **26**, 389 (1961).
7. Monod, J., Wyman, J., and Changeux, J. P., *J. Mol. Biol.*, **12**, 88 (1965).
8. Changeux, J. P., *Brookhaven Symp. Biol.*, **17**, 232 (1964).
9. Jensen, R. A., and Nester, E. W., *J. Biol. Chem.*, **241**, 3365 (1966).
10. Jensen, R. A., and Nester, E. W., *J. Mol. Biol.*, **12**, 468 (1965).
11. Cohen, G. N., *Ann. Rev. Microbiol.*, **19**, 105 (1965).
12. Patte, J. C., Le Bras, G., Loviny, T., and Cohen, G. N., *Biochim. Biophys. Acta*, **67**, 16 (1963).
13. Dixon, M., *Biochem. J.*, **55**, 170 (1953).
14. Rivera, A., and Srinivasan, P. R., Biochemistry, **2**, 1063 (1963).
15. Nester, E. W., and Jensen, R. A., *J. Bacteriol.*, **91**, 1594 (1966).
16. Atkinson, D. E., Hathaway, J. A., and Smith, E. C., *Biochem. Biophys. Res. Commun.*, **18**, 1 (1965).
17. Hill, A. V., *J. Physiol. (London)*, **40**, 190 (1910).
18. Atkinson, D. E., Hathaway, J. A., and Smith, E. C., *J. Biol. Chem.*, **240**, 2682 (1965).
19. Changeux, J. P., *Cold Spring Harbor Symp. Quant. Biol.*, **28**, 497 (1963).
20. Taketa, K., and Pogell, B. M., *J. Biol. Chem.*, **240**, 651 (1965).
21. Caskey, C. T., Ashton, D. M., and Wyngaarden, J. B., *J. Biol. Chem.*, **239**, 2570 (1964).
22. Hochachka, P. W., *Arch. Biochem. Biophys.*, **111**, 96 (1965).
23. Datta, P., Gest, H., and Segal, H., *Proc. Natl. Acad. Sci. U. S.*, **51**, 125 (1964).
24. Srinivasan, P. R., and Sprinson, D. B., *J. Biol. Chem.*, **234**, 716 (1959).
25. Gerhart, J. C., and Schachman, H. K., *Biochemistry*, **4**, 1054 (1965).
26. Cerutti, P., and Guroff, G., *J. Biol. Chem.*, **240**, 3034 (1965).
27. Whiteley, H. R., and Hayaishi, O., *Biochem. Biophys. Res. Commun.*, **14**, 143 (1964).
28. Hathaway, J. A., and Atkinson, D. E., *Biochem. Biophys. Research Commun.*, **20**, 661 (1965).
29. Gerhart, J. C., and Pardee, A. B., *Cold Spring Harbor Symp. Quant. Biol.*, **28**, 491 (1963).
30. Atkinson, D. E., and Walton, G. M., *J. Biol. Chem.*, **240**, 757 (1965).
31. Hirata, M., Tokushige, M., Inagaki, A., and Hayaishi O., *J. Biol. Chem.*, **240**, 1711 (1965).
32. O'Donovan, G. A., and Ingraham, J. L., *Proc. Natl. Acad Sci. U. S.*, **54**, 451 (1965).
33. Rogers, K. S., Geiger, P. J., Thompson, T. E., and Hellerman, L., *J. Biol. Chem.*, **238**, PC481 (1963).
34. Perkins, J., and Bertino, J. R., *Biochem. Biophys. Res Commun.*, **15**, 121 (1964).