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ON THE MECHANISM OF ACTION OF *ESCHERICHIA COLI* TRYPTOPHAN SYNTHASE

STEADY-STATE INVESTIGATIONS

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Summary

Tryptophan synthase from *Escherichia coli* (L-serine hydro-lyase (adding indole), EC 4.2.1.20) synthesizes L-tryptophan from indoleglycerol phosphate and L-serine, releasing glyceraldehyde 3-phosphate, or from indole and L-serine. The latter reaction (B reaction), catalyzed either by the β_2 species or by the $(\alpha_2\beta_2)$ complex, has been studied by steady-state methods. A sequential mechanism is indicated. Inhibition experiments with the substrate analogue benzimidazole were carried out in order to distinguish between random and ordered mechanisms. The results are compatible with a random sequential mechanism. The dissociation constants of the enzyme-substrate complexes are evaluated.

When catalyzed by the tetrameric complex $(\alpha_2\beta_2)$ the B reaction is inhibited by higher concentrations of the substrate indole. This inhibition does not follow the usual substrate inhibition pattern. The question whether the binding of indole to the α-subunit exerts an inhibitory effect on the β_2 species, possibly by reversing the activation by the α subunit of the β_2 species, is discussed.

The enzyme tryptophan synthase (L-serine hydro-lyase (adding indole), EC 4.2.1.20) carries out the last step of the biosynthesis of tryptophan, the synthesis of L-tryptophan from indoleglycerol phosphate and L-serine. This enzyme is a heterotetrameric complex $(\alpha_2\beta_2)$ which is formed by the binding of two α-chains to one β_2 dimer. Each β-chain covalently binds one molecule pyridoxal phosphate forming a Schiff base with the ϵ-amino group of a lysine residue [1].

The $(\alpha_2\beta_2)$ tetramer catalyzes the following reactions [2]:

Indoleglycerol phosphate + L-Serine $\rightleftharpoons$ D-Glyceraldehyde 3-phosphate + L-Tryptophan (1)

Indoleglycerol phosphate ⇌ D-Glyceraldehyde 3-phosphate + Indole (2)

Indole + L-Serine ⇌ L-Tryptophan . (3)

Reaction 1 is catalyzed by $(\alpha_2\beta_2)$, reaction 2 by $(\alpha_2\beta_2)$ or by α, and reaction 3 by $(\alpha_2\beta_2)$ or by β_2.

Other β-replacement reactions or α,β-elimination reactions can be catalyzed by this enzyme also [2—4]. Miles et al. [4] have proposed a scheme comprising the various reactions. The overall reaction (1) is assumed to be the physiological one under normal nutritional conditions.

From the role of α, β_2, or $(\alpha_2\beta_2)$ in Reactions 1—3 it is clear that the isolated α subunits possess active sites for Reaction 2, and β_2 subunits those for Reaction 3. Although there is no proof of this model, one can readily imagine Reaction 1 to consist of Reactions 2 and 3 in sequence. Since no free indole has been detected as an intermediate in the reaction [5,6], the entire sequence of reaction 1 may proceed within a limited space making the loss of the intermediate by diffusion very unlikely.

Assuming that the α- and β-subunits in the complex carry out the same reactions they do when isolated, we have to postulate that Reaction 1 involves a number of unknown isomerization steps, e.g. the transport of an intermediate from a binding site on the α-subunit to that of an adjacent β-chain. In order to avoid these complexities associated with Reaction 1 [7], we started investigating the "B reaction" (3) which is catalyzed by either the isolated β_2 species or the $(\alpha_2\beta_2)$ complex. The activity of the latter is greatly enhanced, compared to the activity of the former.

The investigations were aimed at elucidating the interactions between the indole ligands (tryptophan, indole) and the enzyme proteins and at characterization of the conformational changes in the enzyme triggered by these interactions. To approach these problems the mechanism of the B reaction, especially the way the reactive enzyme · ligand complexes are formed, had to be studied.

Materials and Methods

Enzyme: Both the A and B proteins (α and β_2, respectively) were prepared from cells of an *E. coli* W3110 strain (Heilmann, H.-D., unpublished) producing large amounts of the $(\alpha_2\beta_2)$ complex and an excess of the α subunit. This *E. coli* mutant was kindly provided by Dr. C. Yanofsky. When it was necessary to use B protein absolutely free of traces of A protein, it was prepared [9] from cells of an *E. coli* mutant $(A_2/F'A_2)$ which cannot synthesize intact A protein (donated by Dr. I.P. Crawford).

All reagents used were analytical grade or highest purity commercially available. Indole and benzimidazole were recrystallized prior to use. The enzymatic activity was assayed by recording the absorbance change at 289.9 nm in a Gilford 240 recording spectrophotometer. The linearity between the concentration change and instrument reading was checked. The incubation mixture for the assays was prepared according to the method of Smith and Yanofsky [9] except that the concentrations of the substrates, indole and serine, were changed as desired.

When the activity in the B reaction of the $(\alpha_2\beta_2)$ complex was assayed, the

concentration of the α subunit was at least four times that of the β subunit to make sure that all activity was due to the complex [5].

Slopes and intercepts of the curves were calculated by linear regression analysis. Activities are expressed as units according to the system of Smith and Yanofsky [9]. 1 unit (YU) = $8.33 \cdot 10^{-11}$ katal; it signifies the amount of enzyme that causes the disappearance of 0.1 μmol indole in 20 min under the conditions described [9]. The specific activity of the B protein used was at least 2000 YU/mg in the presence of A protein. Between 0.3 and 4 YU of $\alpha_2\beta_2$ or β_2, respectively, were added to 1 ml incubation mixture in the cuvettes.

Results

1. Steady-state experiments

The activity of the enzyme was evaluated as a function of the concentration of either substrate, indole or L-serine, keeping the concentration of the second substrate constant. Fig. 1 shows the results for the ($\alpha_2\beta_2$) complex in double-reciprocal plots. When the initial reaction rate is plotted reciprocally against the reciprocal indole concentration, all curves intersect at one point in the lower left quadrant (Fig. 1a). The same pattern is obtained with the reciprocal L-serine concentration plotted on the abscissa, with indole concentrations up to 0.03 mM (Fig. 1b). With higher indole concentrations all curves intersect on the ordinate (Fig. 1c). With the reciprocal reaction rate plotted against the indole concentration [10] a straight line should emerge in the case of a competitive inhibition exerted by indole. However, the experimental curve is not linear as shown in Fig. 2. Thus a simple substrate inhibition model or a competition mechanism between indole and L-serine at the serine binding site may be ruled out. The same result was obtained from a secondary plot [11] of the slopes of the primary plots (Fig. 1) against the indole concentration as shown in Fig. 3.

When similar experiments were carried out using β_2 instead of ($\alpha_2\beta_2$) the same pattern was observed in general (Fig. 4a, b) except that there was no inhibition by indole in the entire concentration range investigated (up to 1 mM).

Figs. 5 and 6 show secondary plots of the intercepts and slopes, respectively, of the primary plots against the reciprocal substrate concentrations. They yield the Michaelis constants of the different complexes (enzyme · indole), (enzyme · serine), and (enzyme · indole · serine) (Table I).

2. Inhibition by substrate analogues

In order to characterize further the mechanism of the B reaction the inhibition by a substrate analogue was studied. Table II shows the inhibition of the B reaction exerted by a number of substances structurally related to indole. The reaction product L-tryptophan (not shown in the table) does not significantly inhibit the reaction when administered in concentrations up to 1 mM. In this experiment the indole concentration varied between 0.01 and 0.3 mM.

The figures given in Table II indicate benzimidazole to be the most efficient inhibitor of the substances tested. Fig. 7 shows that the difference spectrum of the binding of benzimidazole to β_2 rather closely resembles the difference spectrum of indole binding in the double peaks and the single trough but the absorption maxima are displaced.

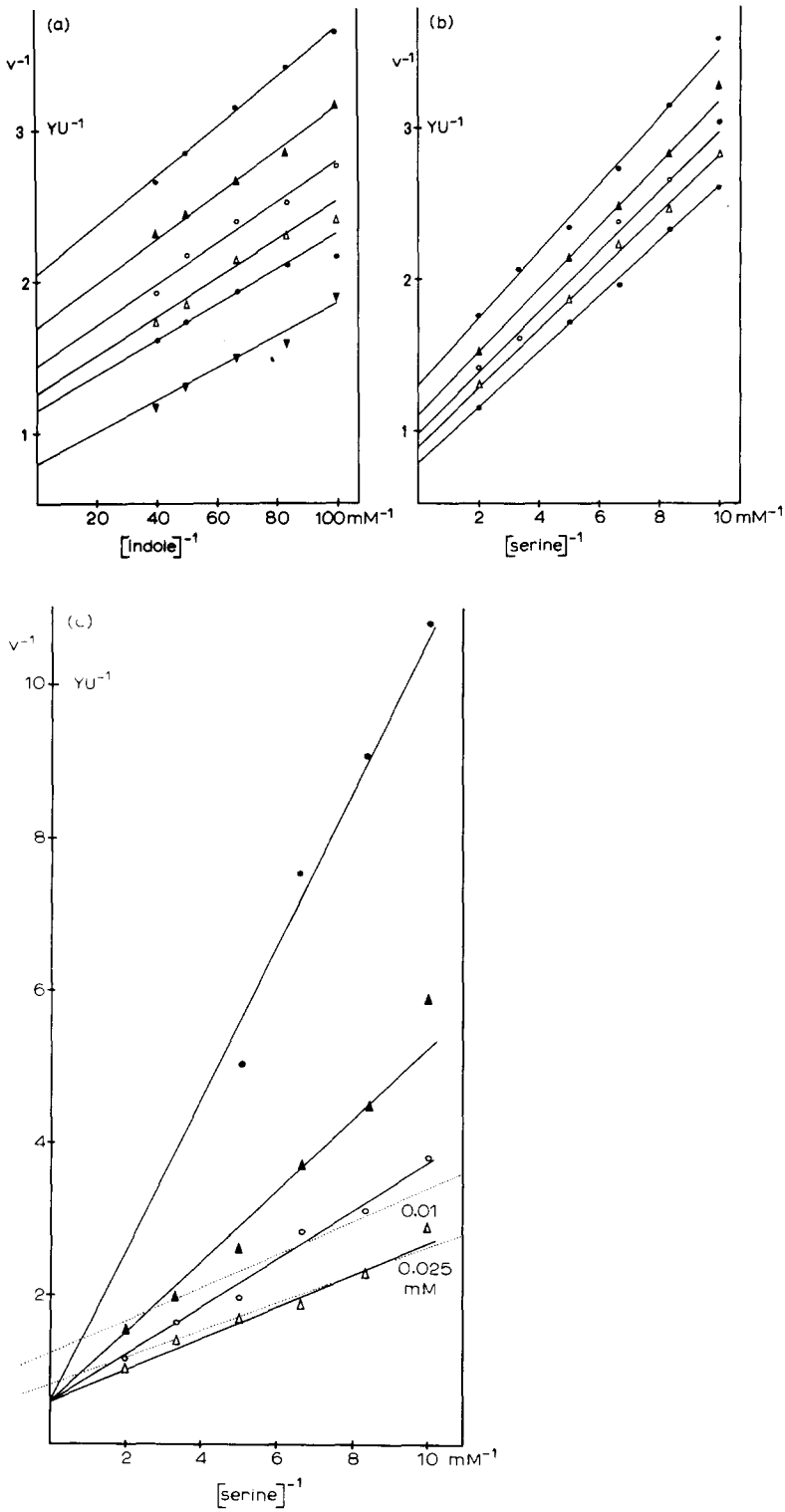
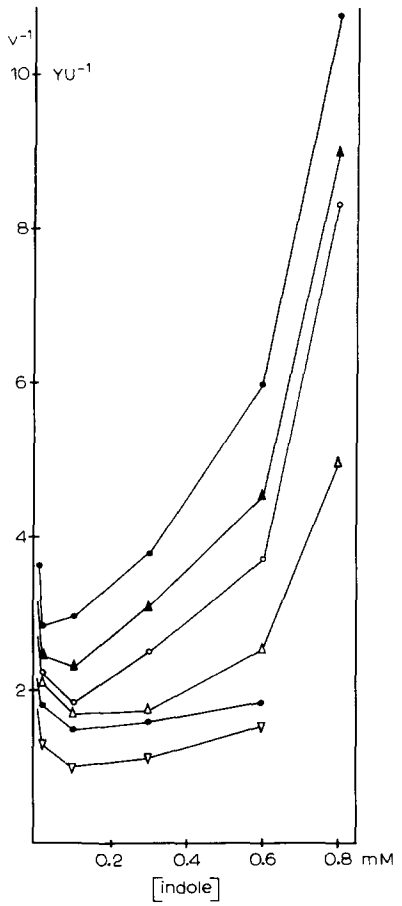


Fig. 1. Double reciprocal plot of the reaction rate in the B reaction vs. substrate concentrations. Enzyme: ($\alpha_2\beta_2$). a. Abscissa; [indole]$^{-1}$; [L-serine] = 0.1, 0.12, 0.15, 0.2, 0.3, 0.5 mM (from top to bottom). b. Abscissa: [L-serine]$^{-1}$; [indole] = 0.01, 0.012, 0.015, 0.02, 0.025 mM (from top to bottom). c. Abscissa: [L-serine]$^{-1}$; solid lines: [indole] = 0.8, 0.6, 0.3, 0.1 mM (from top to bottom); dotted lines: [indole] = 0.01, 0.025 mM.



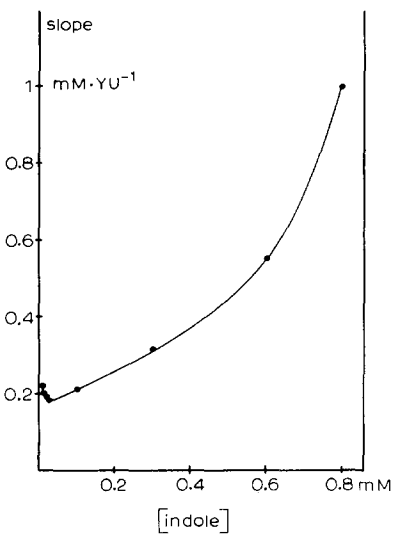


Fig. 2. Plot of reciprocal reaction rate vs. indole concentration. [L-serine] = 0.1, 0.12, 0.15, 0.2, 0.3, 0.5 mM (from top to bottom).

Fig. 3. Plot of the slopes of the curves in Figs. 1b and 1c vs. the indole concentration.

The pattern of inhibition by benzimidazole of the B reaction was investigated by varying the concentrations of indole and benzimidazole, the L-serine concentration being held constant at 10 mM. Fig. 8 shows the double-recipro-

TABLE I

MICHAELIS CONSTANTS OF THE ENZYME-SUBSTRATE COMPLEXES AT 307 K (β_2) OR 309 K ($\alpha_2\beta_2$)

E = Enzyme, S = L-Serine, I = Indole.

Reaction	Michaelis constant	Values of the constants (μM)	
		for β_2	for $\alpha_2\beta_2$
E + S ⇌ ES	K'_A	94	77
E + I ⇌ EI	K'_B	4.0	3.7
EI + S ⇌ ESI	K_A	414	313
ES + I ⇌ ESI	K_B	15	14

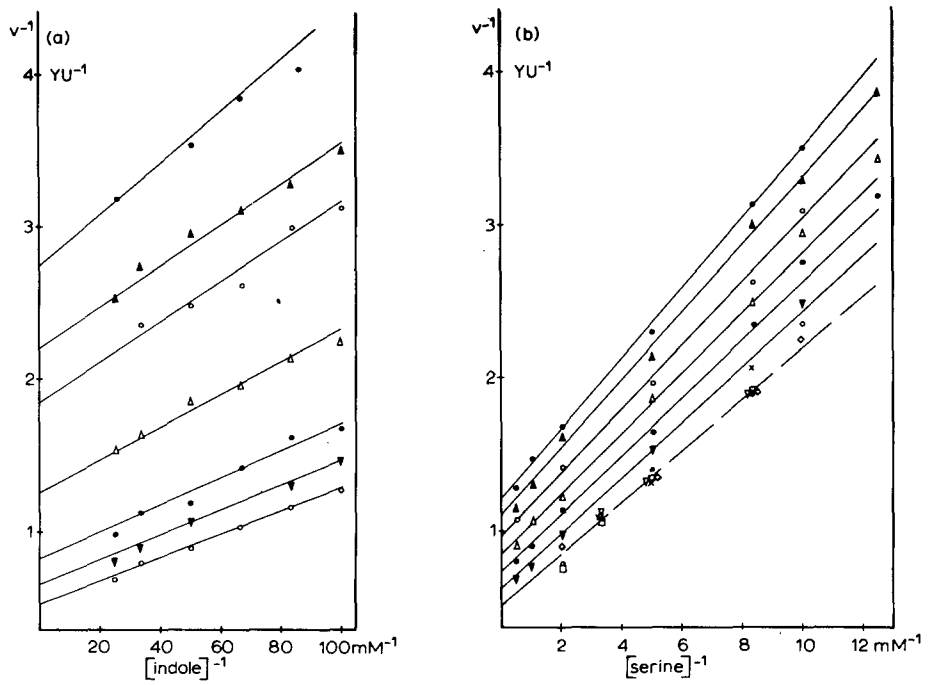


Fig. 4. Double reciprocal plot of the reaction rate in the B reaction vs. substrate concentrations. Enzyme: β_2. a. Abscissa: $[\text{indole}]^{-1}$; [L-serine] = 0.08, 0.1, 0.12, 0.2, 0.5, 1, 2 mM (from top to bottom). b. abscissa: $[\text{L-serine}]^{-1}$; [indole] = 0.01, 0.012, 0.015, 0.02, 0.03, 0.04 mM (from top to bottom, solid line), dashed line: 0.1 (○), 0.3 (□), 0.6 (◇), 0.8 (▽), 1.0 (×) mM.

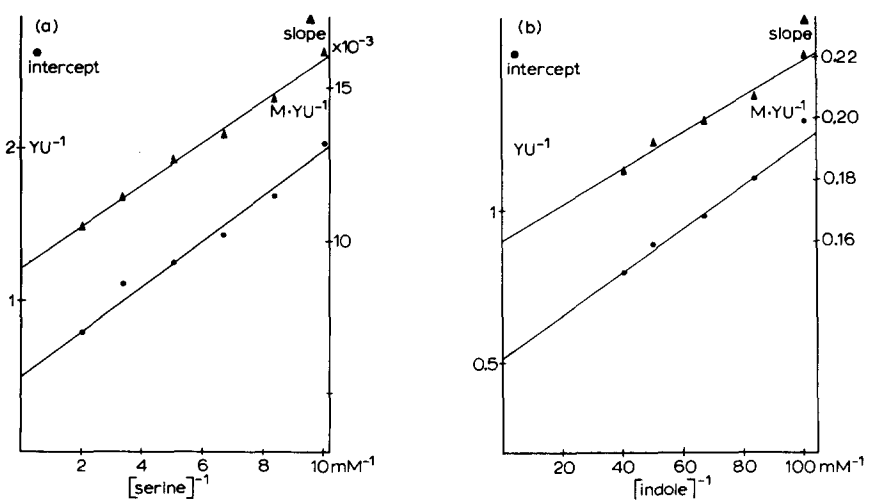


Fig. 5. Secondary plots of slopes and intercepts of the primary plots (Figs. 1a and 1b) vs. reciprocal substrate concentrations. Enzyme: $\alpha_2\beta_2$. The constants K_A, K_B, K'_A K'_B are calculated from the intercepts on the ordinate and the slopes of these plots [11]: a: intercepts (●): intercepts = $1/V$, slope = K_A/V; slopes (▲): intercept = K_B/V, slope = $K'_AK_B/V = K_AK'_B/V$; b: intercepts (●): intercept = $1/V$, slope = K_B/V; slopes (▲): intercept = K_A/V, slope = K'_AK_B/V.

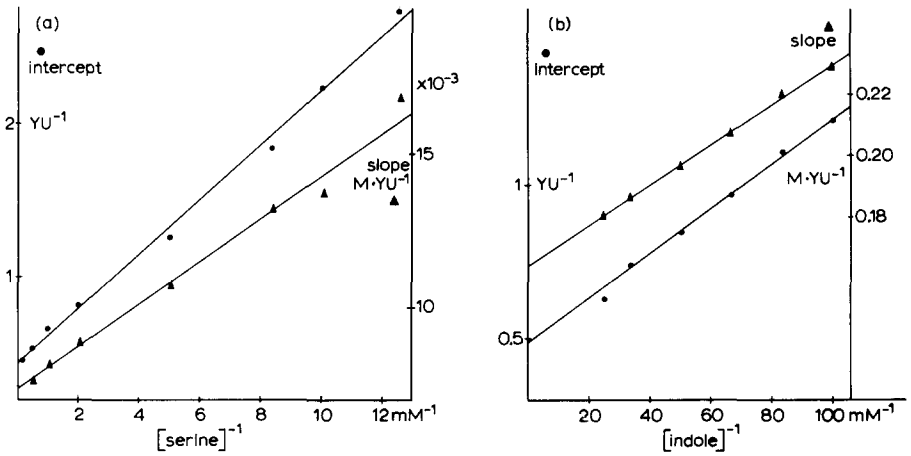


Fig. 6. Secondary plots of slopes and intercepts of the primary plots (Figs. 4a and 4b) vs. reciprocal substrate concentrations. Enzyme: β_2. For the calculation of the constants see the legend to Fig. 5.

TABLE II

INHIBITION OF THE B REACTION BY SUBSTRATE ANALOGUES

Concentrations: [L-serine] = 10 mM, [indole] = 0.03 mM, [inhibitor] = 1 mM.

Inhibitor	Enzyme	Inhibition (%)
Indazole	$\alpha_2\beta_2$	27
	β_2	15
Indolepropionic acid	$\alpha_2\beta_2$	37
	β_2	37
Purine	$\alpha_2\beta_2$	5
	β_2	1
Benzimidazole	$\alpha_2\beta_2$	72
	β_2	71
L-tryptophan methyl ester	$\alpha_2\beta_2$	10
	β_2	0

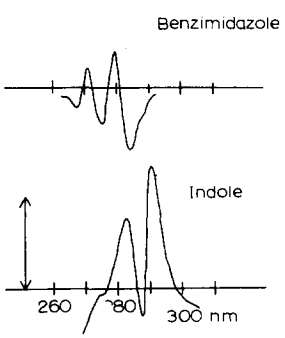


Fig. 7. Difference spectra of the binding of indole and benzimidazole to β_2. Spectra were recorded at room temperature with a Cary 15 spectrophotometer using twin cells, optical path length 0.47 cm per chamber. Concentrations: enzyme, $2.5 \cdot 10^{-5}$ M, ligands, $2 \cdot 10^{-4}$ M. Ordinate: Length of the bar indicates 0.01 absorbance units.

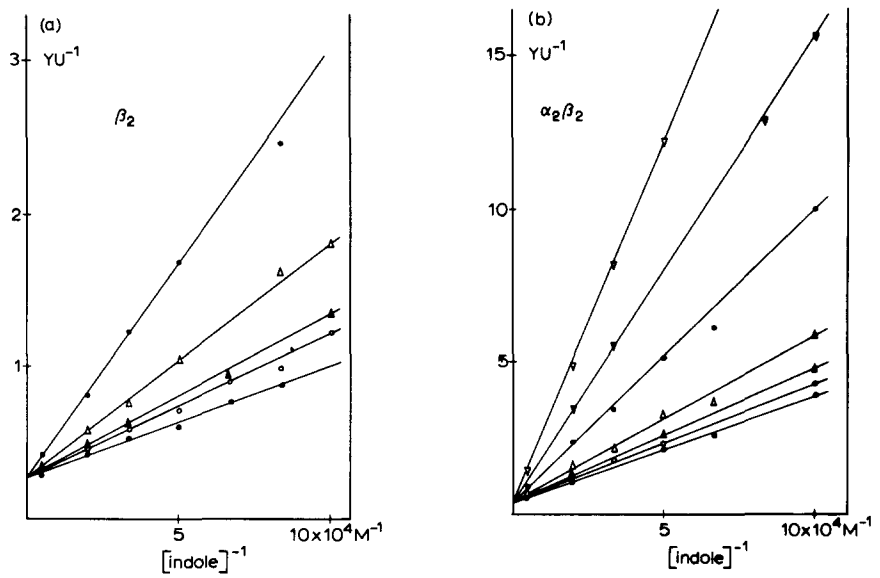


Fig. 8. Inhibition by benzimidazole of the B reaction. Double reciprocal plot of the reaction rate vs. indole concentration. a. Enzyme: β_2; [benzimidazole] = 0.3, 0.1, 0.03, 0.01, 0 mM (from top to bottom). b. Enzyme: ($\alpha_2\beta_2$); [benzimidazole] = 2, 1, 0.3, 0.1, 0.03, 0.01, 0 mM (from top to bottom).

cal plots of the reaction rate versus the indole concentration. Both β_2 (Fig. 8a) and ($\alpha_2\beta_2$) (Fig. 8b) were tested. In each case the curves for different inhibitor concentrations intersect at one point on the ordinate.

Discussion

The elucidation of the mechanism of the tryptophan synthase reactions has been the aim of several steady-state and stopped-flow or T-jump experiments [7,12—14], spectroscopic [15] and equilibrium dialysis tests [16]. A kinetic mechanism for the conversion of indoleglycerol phosphate and L-serine to tryptophan and glyceraldehyde 3-phosphate (Reaction 1) was postulated by Creighton [7]. Faeder and Hammes [12,13] investigated the B reaction. They found that both a compulsory sequence of substrate addition, and a rapid random equilibration between enzyme and substrate are consistent with the results of their rapid reaction and steady-state experiments.

The contribution presented here describes the assay of the B reaction of the *E. coli* enzyme under steady-state conditions. When the reaction rate data were treated as shown in Figs. 1 and 4—6, the pattern obtained was clearly indicative of a rapid equilibrium sequential mechanism. On the basis of these steady-state experiments, however, one cannot distinguish between an ordered or a random sequential mechanism [17]. To discern between the mechanisms in question the inhibition by benzimidazole as substrate analogue was studied by plotting the reaction rate double reciprocally against the indole concentration. In the case of an ordered ternary complex mechanism the rate equation in the recipro-

cal form [11] reads

$$\frac{V}{v} = \frac{1}{[B]}\left[K_B\left(1 + \frac{[Q]}{K_Q^A}\right) + \frac{K'_A K_B}{[A]}\left(1 + \frac{[Q]}{K_Q}\right)\right] + \frac{K_A}{[A]}\left(1 + \frac{[Q]}{K_Q}\right) + 1 .$$

When the reciprocal reaction rate is plotted against the reciprocal indole concentration ($[B]^{-1}$), the intercepts on the ordinate of the curves for different inhibitor concentrations ([Q]) vary with [Q] as indicated by the term $K_A/[A]$ $(1 + [Q]/K_Q) + 1$. (It should be mentioned that the meaning of K_A as it is used here is different from its meaning in the following equation where it signifies the Michaelis constant of the formation of the (enzyme · indole · serine) complex from the (enzyme · indole) complex and L-serine.)

On the other hand, the random mechanism depicted in Fig. 9 yields the following reciprocal rate equation [12]:

$$\frac{V}{v} = \frac{1}{[B]}\left[K_B\left(1 + \frac{[Q]}{K_Q^A}\right) + \frac{K'_A K_B}{[A]}\left(1 + \frac{[Q]}{K_Q}\right)\right] + \frac{K_A}{[A]} + 1 .$$

Plotted as above, the intercept is independent of [Q] and the curves intersect on the ordinate. Fig. 8 shows that the inhibition pattern is compatible with a random sequential mechanism (Fig. 9). This scheme applies both to the $(\alpha_2\beta_2)$ and to the β_2 species.

The steady-state experiments yield the Michaelis constants for the different enzyme · substrate complexes, determined from the secondary plots (Figs. 5 and 6). Table I shows the constants for each step of the mechanism as depicted in Fig. 9. The order of magnitude of the Michaelis constant of the (β_2 · indole) complex agrees with the dissociation constant obtained from equilibrium dialysis experiments carried out at very low enzyme concentrations [19].

High indole concentrations inhibit the activity of the $(\alpha_2\beta_2)$ complex in the B reaction. Although from the double reciprocal plot the inhibition appears to be competitive with respect to serine, the true nature of the inhibition is neither competitive nor of the substrate inhibition type. The following statements can be made concerning this inhibition by indole:

1. Indole concentrations above 0.03 mM inhibit the activity in the B-reaction of the $(\alpha_2\beta_2)$ complex, not that of the β_2 species.

2. Binding of α-subunits to the β_2 species causes activation of the complex in the B-reaction.

3. Indole binds to the α-subunit (K_d values of the (α · indole) complexes =

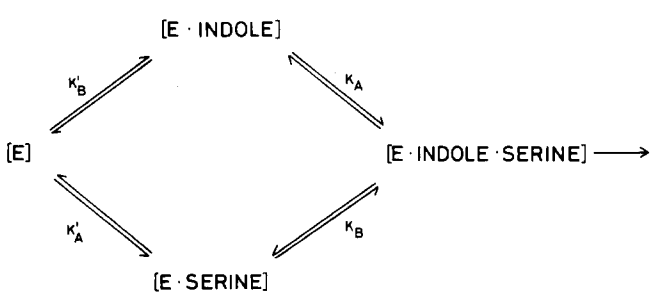


Fig.9. Random sequential mechanism of the B reaction of tryptophan synthase.

18 and 1.5 mM as determined by Weischet and Kirschner [16]). Thus at indole concentrations which bring about inhibition in the B reaction of the $(\alpha_2\beta_2)$ complex there is considerable binding of indole to the α-subunit. For example, with 0.04 mM indole already 2.6% of α subunits are in the form of (α · indole) complex as calculated using K_d = 1.5 mM. At higher indole concentrations the interaction between the two types of indole binding sites [19] must be taken into consideration.

These observations, some of which are well-known for this system, agree with the assumption that binding of indole to the α subunit exerts a certain transconformational effect upon the β subunit via subunit interaction. This effect appears to be inhibitory to the B reaction. There is no evidence so far that binding of indole by β_2 can cause this inhibition. Furthermore, any sort of mutual influence of the ligand binding at both active sites on one β_2 unit tends to be refuted by the observation that the Hill coefficient for the β_2-catalyzed reaction is close to unity (Fig. 10).

It is not evident from these studies whether or not the phenomenon involves a reversal of the activation by the α subunit of the β_2 species. Besides the models mentioned, other models are conceivable which will fit the experimental data for the inhibition by high indole concentrations. We are now studying the effect in more detail in order to distinguish between the different possibilities.

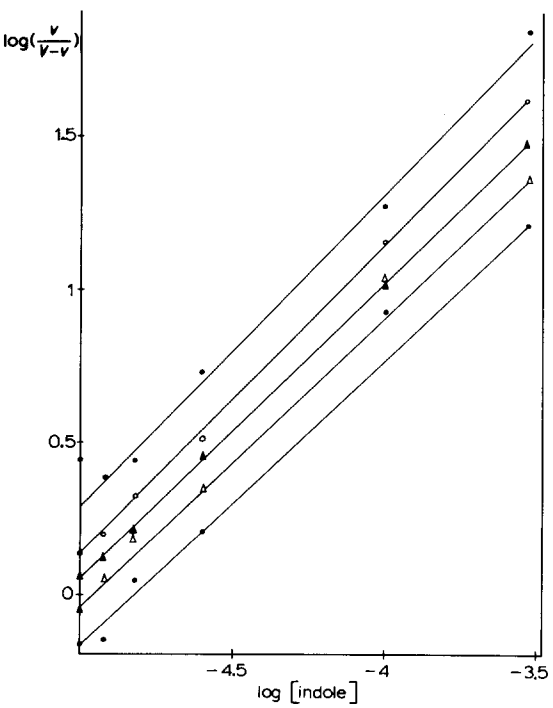


Fig. 10. Hill plot. Enzyme: β_2. [L-serine] = 0.08, 0.12, 0.20, 0.30, 0.50 mM (from top to bottom).

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