Indole Channeling by Tryptophan Synthase of Neurospora*

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SUMMARY

Tryptophan synthase (L-serine hydro-lyase-adding indole) (EC 4.2.1.20) of Neurospora crassa was studied in reaction mixtures containing [2-14C]indoleglycerolphosphate and nonlabeled indole. A detailed kinetic analysis of the passage of indole from indoleglycerolphosphate to indole and to tryptophan during the synthesis of tryptophan revealed that: (a) indole disappears first and indoleglycerolphosphate is utilized after the indole concentration falls to barely detectable levels; (b) significant amounts of radioactivity appear in indole; and (c) the specific radioactivity of the tryptophan formed is much lower than that of indoleglycerolphosphate but 2- to 5-fold higher than that of indole depending inversely upon the initial indole concentration. The analyses of these observations are interpreted as evidence that indole is an enzyme-bound or "channeled" intermediate in the conversion of indoleglycerolphosphate to tryptophan. The enzyme was separated from reaction mixtures containing an equilibrium mixture of labeled indoleglycerolphosphate and labeled indole by passage of the mixtures through ultrafilters which retained material of molecular weights greater than 50,000. Radioactive indole was found exchangeably bound to the retained enzyme. From these results, it was estimated that 2 to 3 moles of indole were bound per mole of enzyme. The results are interpreted as evidence that the Neurospora enzyme fits well the conceptual framework of the "surface model" (Davis, R. H. (1967) in *Organizational Biosynthesis* (Vogel, H. J., Lampen, J. O., and Bryson, V., eds) pp. 303-322, Academic Press, New York) and that the enzyme may have evolved, as first suggested (Bonner, D. M., DeMoss, J. A., and Mills, S. E. (1965) in *Evolving Genes and Proteins* (Bryson, V., and Vogel, H. I., eds) pp. 305-318, Academic Press, New York), from a primitive multi-component complex.

1. Pyridoxal phosphate is bound to the enzyme at Site II. Serine also binds at this site through formation of a Schiff base with enzyme-bound pyridoxal phosphate.
2. For catalysis of Reaction 1, indoleglycerolphosphate binds at Site I of the enzyme-pyridoxal phosphate-serine complex. Glyceraldehyde 3-phosphate is liberated and the indole moiety of indoleglycerolphosphate is transferred to Site II. At Site II a condensation occurs between indole and the reactive carbon skeleton of serine which apparently involves the prior dehydration of the serine molecule (2). After condensation, tryptophan and free enzyme are liberated.
3. For catalysis of Reaction 2, indole is bound to Site II of the enzyme-pyridoxal phosphate-serine complex and dehydration and condensation occur as above.
4. For catalysis of Reaction 3f, indoleglycerolphosphate is bound to resolved enzyme1 at Site I. Indole and glyceraldehyde 3-phosphate are liberated from the enzyme. In the reverse Reaction 3r, indole and glyceraldehyde 3-phosphate are bound at Site I, and indoleglycerolphosphate and free enzyme are generated.

1 Resolved enzyme is apotryptophan synthase which has been freed of pyridoxal phosphate and serine by treatment with hydroxylamine (1).
phosphate was located (Rp, 0.09) with a hand-held radiation detector.

The radioactive indoleglycerolphosphate was partially deproteinized by immersion in a boiling bath for 2 min. The sample was then chilled and delivered into a tube immersed in a boiling water bath. The tube was capped, and the reaction was terminated by boiling for 2 min. A sample (1.0 ml) was withdrawn and delivered into a tube immersed in a boiling bath. The tube was capped, and the reaction was terminated by boiling for 2 min.

The radioactive indoleglycerolphosphate was isolated from the reaction mixture by ascending chromatography on Whatman No. 3MM filter paper by development in a solvent system consisting of isopropanol:ammonia:water (300:5:5:5). One milliliter of the deproteinized and tolueno-extracted reaction mixture was applied to each of six sheets of filter paper (42 × 50 cm). The radioactive indoleglycerolphosphate was located (Rp, 0.09) with a hand-held radiation detector.

The radioactive material was eluted from the paper with hot ammoniacal ethanol, and the resulting solution was concentrated in vacuo. This material co-chromatographed with authentic indoleglycerolphosphate. In control experiments, it was used as substrate in reaction mixtures containing excess tryptophan synthase, serine, and pyridoxal phosphate. The amount of tryptophan formed in such reaction mixtures was stoichiometrically dependent upon the amount of indoleglycerolphosphate added. The specific radioactivity of the tryptophan formed was equal to the indoleglycerolphosphate supplied.

Unlabeled indoleglycerolphosphate was prepared as previously described (2), except that a chromatographic procedure described by Lester (4) was employed in place of the barium precipitation step. Using this method, indoleglycerolphosphate is well resolved from indoleglycerol and other materials which absorb at 280 nm.

**Methods**

**Organisms and Extracts**—Partially purified tryptophan synthase prepared from extracts of wild type *N. crassa* (strain 74A) was employed in this work. The methods of purification and some properties of the preparation have been described previously (2).

**Preparation of Substrates**—Radioactive indoleglycerolphosphate was prepared enzymically from [2-14C]indole and glyceraldehyde 3-phosphate. The reaction mixture (6.0 ml) contained [2-14C]indole (specific radioactivity 8.4 &mu;Ci per pmole), 5 mM; glyceraldehyde 3-phosphate; Trp, L-tryptophan; Ind, indole; GAP, glycer-aldehyde 3-phosphate; H@P, pyridoxal phosphate; Ser, L-serine.

The experiments described in this report are an extension of earlier observations (2) on the inhibition of tryptophan synthase by indoleacrylic acid. This compound exerts its inhibition by interfering with the binding of serine at Site II. The inhibitor is thus a probe which magnifies the catalytic contribution of Site I relative to Site II in reactions of the enzyme which might require the function of both Sites I and II (i.e. Reaction 1). In studies described here, the enzyme, in the presence and absence of inhibitor, was presented with saturating quantities of indoleglycerolphosphate labeled with 14C in Position 2 of the indole moiety. The reaction mixtures also contained various levels of nonlabeled indole. The kinetics of appearance of isotope in both indole and tryptophan were determined by sampling the reaction mixtures at appropriate intervals during net synthesis of tryptophan. From these measurements, the argument is developed that indole is a channeled intermediate in the conversion of indoleglycerolphosphate to tryptophan, and further, it is confined to a microenvironment at or near the catalytic surfaces of the enzyme.

**RESULTS**

Tryptophan synthase in the presence of pyridoxal phosphate will catalyze the formation of tryptophan from indoleglycerolphosphate and serine. If indole is also present in the reaction mixture, the rate of appearance of tryptophan is considerably higher. In such a mixture, indole is utilized first and indole glycerolphosphate is utilized only after the concentration of indole has fallen to barely detectable levels (1). Fig. 2 presents data obtained from 10 of 18 separate reaction mixtures each of which received, initially, a saturating concentration (0.55 mM) of [2-14C]indoleglycerolphosphate (specific radioactivity ~400 nCi per pmole). These reaction mixtures also received, initially, concentrations of indole varying from 0 to 2.64 &mu;mM. Reaction mixtures *F* through *J* received the inhibitor indoleacrylic acid at a level (0.35 mM) which gives about 50% inhibition of Reactions 1 and 2 of tryptophan synthase (2). The inhibited reactions received twice the amount of enzyme so as to adjust the rate of appearance of tryptophan to a level approximately equal to that of the uninhibited reactions (A through E). Each reaction mixture was sampled at intervals of 10 min. A sample (1.0 ml) was withdrawn and delivered into a tube immersed in a boiling water bath. The tube was capped, and the reaction was terminated by boiling for 2 min. The sample was then chilled and subjected to the following assay procedures: (a) total indole as Ehrlich-positive material extractable into toluene; (b) radioactivity in indole (see “Methods”); (c) total tryptophan in the aqueous layer from a as Ehrlich-positive material extractable into toluene over a tryptophanase digest (1); (d) radioactivity in tryptophan as in b above; (e) total indoleglycerolphosphate in the aqueous layer from c as material absorbing at 290 nm extractable into ethylacetate after oxidation with sodium metaperiodate (1); (f) radioactivity in indoleglycerolphosphate as radioactivity in the ethylacetate layer from e.
FIG. 2. Formation of tryptophan in reaction mixtures containing radioactive indoleglycerolphosphate and various concentrations of nonradioactive indole. X——X, specific radioactivity of tryptophan-precursor indole; O——O, specific radioactivity of indoleglycerolphosphate; ■——■, indoleglycerolphosphate (nm); □——□, specific radioactivity of tryptophan; n——n, specific radioactivity of solvent indole; ▲——▲, solvent indole (nm). All reaction mixtures contained the following: potassium phosphate, 50 mM, pH 7.8; pyridoxal phosphate, 0.2 μM; t-serine, 40 mM; indoleglycerolphosphate, 0.5 mM (specific radioactivity ~400 nCi per μmole); and 0.01 Reaction 2 units of tryptophan synthase per ml of reaction mixture. Reactions B through E and G through J received nonradioactive indole as follows: I and G, 0.4 mM; C and H, 0.6 mM; D and I, 0.8 mM; and E and J, 1.0 mM. Reactions F through J received indolacrylic acid, 0.35 mM, and twice the amount of tryptophan synthase. Other experimental procedures are detailed in the text. Note scale changes and interruptions on right ordinates. Tabular data taken from 18 such reaction mixtures (10 of which are described in this figure) have been supplied for review and deposit by the Editors.*

The characteristics of tryptophan formation by the enzyme when it is presented with both indoleglycerolphosphate and indole are apparent in the data obtained from reaction mixtures A through E. In A, tryptophan formation proceeded at a constant rate from indoleglycerolphosphate. In B through E tryptophan formation proceeded at nearly a 2-fold higher rate. In these mixtures indole was utilized early and the concentration of indoleglycerolphosphate remained essentially constant until the concentration of indole fell to somewhat less than 0.1 mM.

Fig. 3 presents the kinetics of passage of isotope from indoleglycerolphosphate into indole and tryptophan in the reaction mixtures described in Fig. 2. Reaction mixtures A through E of Fig. 2 received initial indole concentrations varying from 0 to 1.0 mM. Reaction mixtures F through J of Fig. 2 received the same range of initial indole concentrations and in addition they received 0.35 mM indolacrylic acid. Panels A and B of Fig. 3 show the time course of appearance of total radioactive indole. Panels C and D show the time course of appearance of radioactive tryptophan. Panels B and D describe reaction mixtures which received indolacrylic acid (IA) (0.35 mM). ■——■, no added indole (Fig. 2A); □——□, initial indole (0.4 mM) (Fig. 2B); O——O, initial indole (0.6 mM) (Fig. 2C); O——O, initial indole (0.8 mM) (Fig. 2D); X——X, initial indole (1.0 mM) (Fig. 2E).

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3 Supplementary data taken from 18 separate reaction mixtures, 10 of which are described in Fig. 2, are available as JBC Document Number 73M-1680, in the form of one microfiche or 5 pages. Orders for supplementary material should specify the title, author and reference to this paper and the JBC Document number, the form desired (microfiche or photocopy), and the number of copies desired. Orders should be addressed to The Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014 and must be accompanied by remittance to the order of the Journal in the amount of $2.50 for microfiche or for photocopy.

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FIG. 3. Rates of entry of total isotope into indole and tryptophan. These data were taken from the reaction mixtures described in Fig. 2. Panels A and B show the time course of appearance of total radioactivity in indole. Panels C and D show the time course of appearance of radioactivity in tryptophan. Panels B and D describe reaction mixtures which received indolacrylic acid (IA) (0.35 mM). ■——■, no added indole (Fig. 2A); □——□, initial indole (0.4 mM) (Fig. 2B); O——O, initial indole (0.6 mM) (Fig. 2C); O——O, initial indole (0.8 mM) (Fig. 2D); X——X, initial indole (1.0 mM) (Fig. 2E).
indoleglycerolphosphate entered tryptophan was markedly reduced. This result was, of course, expected from the well known early utilization of indole reported previously (1). Of equal or even greater interest was the observed trend in the rate of appearance of radioactivity in indole in the reaction mixtures. This rate increased with increasing concentrations of indole. The indole initially present appeared to act with increasing effectiveness as a trapping pool for isotope passing from indoleglycerolphosphate to tryptophan. Thus, indole in this reaction seems to exhibit one of the traditional criteria of an intermediate in an enzymically mediated conversion.

One of the original arguments employed in ruling out the participation of Reaction 3f (and therefore of indole as an intermediate) in the conversion of indoleglycerolphosphate to tryptophan was the observation that Reaction 3f proceeded at an apparent rate 50-fold lower than that required to sustain Reaction 1. Analysis of the rates presented in Panels B and D of Fig. 3 bears on this point. The reaction mixtures described in these panels contained 0.35 mM indoleacrylic acid, an inhibitor, which for the reasons stated above, would tend to magnify the catalytic activity of Site I relative to Site II in the context of the DeMoss model. In these tubes the ratios (InGl' + Trp/InGl' + Ind) (where InGP is indoleglycerolphosphate, Trp is L-tryptophan, and Ind is indole) were observed to vary over a 100-fold range with increasing concentrations of initially supplied indole. As shown in Panel B of Fig. 3, the initial rates of appearance of isotope in indole were measurably higher than those shown in Panel A, while the rates of isotope entry into tryptophan (Panel D) were nearly equal to those shown in Panel C. For each initial concentration of indole, consequently, the ratio (InGP → Trp/InGP → Ind) of these rates was somewhat lower, indicating about a 2-fold higher relative activity of catalysis at Site I in the presence of indoleacrylic acid.

Fig. 4 presents the results of a similar analysis of the data taken from reaction mixtures A through E and additional reaction mixtures which had received increasing initial concentrations of indole (see legend to Fig. 2). In this figure the ratio of the initial rate of entry of isotope into tryptophan to the rate of entry of isotope into indole is plotted on the ordinate as a function of the initial concentration of indole. The implication of this analysis is that indole initially present in the reaction mixture served as a partially effective trapping pool for isotope passing from indoleglycerolphosphate to tryptophan and therefore that indole or a compound freely exchangeable with indole is an intermediate in this reaction.

The trapping phenomenon argues favorably for the identity of indole as an intermediate. Of equal or even greater significance is the observed efficiency of trapping indicated in Fig. 4. It should be recalled (cf. Fig. 1) that the $K_m$ of Site II for indole is $\sim 0.1$ mM. In usual practice 0.5 mM indole is regarded as a saturating concentration with respect to Reaction 2. As shown in Fig. 4, five times this concentration was required for essentially complete trapping. The observed lack of efficient trapping argues favorably for the concept that indole arising as a result of catalysis at Site I is channeled to and preferentially used by Site II. These results are formally similar to those reported by Lue and Kaplan (5) for the carbamyl phosphate synthase-aspartate-transcarbamylase aggregate.

Clearly, these results cannot exclude the possibility that an as yet unknown compound is an intermediate in the conversion of indoleglycerolphosphate to tryptophan and that this compound is in equilibrium with indole present in the reaction mixture. On the other hand, they offer no compelling reason to invoke the existence of such a compound. An alternative view which appears consistent with these results is the surface model originally presented by Davis in a much cited paper (6). According to this concept indole generated as a result of catalysis at Site I would enter a space near the catalytic surface of the enzyme. The relatively high affinity of Site II for indole and the inability of Site I to produce saturating levels of indole (because of its lower $V_{max}$) practically ensures that indole would behave as an enzyme-bound or channeled intermediate. In this case an abnormally high local concentration of free indole in the solvent would be expected to exchange with indole present in the "channel." If the channeled indole were labeled with radioactivity, the radioactivity would thus be "unchanneled" and would appear in the free indole in the solvent. The effect noted in Fig. 4 could thus be conveniently interpreted as a case of essentially complete "unchanneling" at indole concentrations in the solvent of 2.0 to 2.5 mM.

If the surface model of Davis (6) is applicable to this system, it seems reasonable to suppose that the enzyme would catalyze an exchange reaction involving the indole moiety of indoleglycerolphosphate and free indole dissolved in the solvent. It was of interest, therefore, to study the question of exchange of the indole moiety of indoleglycerolphosphate with free indole in the presence of the enzyme. Although this question has been studied before (1, 7), and negative results obtained, a reconsideration seemed indicated in view of the fact that indole appeared to be a concentration-dependent trapping agent for isotope originating in indoleglycerolphosphate. For this purpose a preparation of "resolved enzyme" (1, 2) was employed. For this experiment, which is described in Table I, the basic reaction mixture contained equimolar concentrations of labeled indoleglycerolphosphate and unlabeled indole. If pyridoxal phosphate and serine were added (tube 4), the disappearance of indole and appearance of tryptophan were readily observed. Ra-

![Fig. 4. Influence of initial concentration of indole on its ability to trap isotope passing from indoleglycerolphosphate to tryptophan. The ratio of the initial rate of appearance of isotope in tryptophan to the initial rate of appearance of isotope in indole (cf. Fig. 3) is plotted as a function of the initial concentration of indole in the reaction mixture. For methods see Fig. 2.](http://www.jbc.org/content/241/1/4044/F4)

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[Figure 4](http://www.jbc.org/content/241/1/4044/F4) shows the influence of initial concentration of indole on its ability to trap isotope passing from indoleglycerolphosphate to tryptophan. The ratio of the initial rate of appearance of isotope in tryptophan to the initial rate of appearance of isotope in indole (cf. Fig. 3) is plotted as a function of the initial concentration of indole in the reaction mixture (Fig. 4). The data presented indicates that indole reacts with increasing effectiveness as a trapping pool for isotope passing from indoleglycerolphosphate to tryptophan. This increase in efficiency is observed primarily at lower indole concentrations, with the ratio of initial rates increasing with increasing indole concentration. The study further suggests that indole may act as an intermediate in the conversion of indoleglycerolphosphate to tryptophan.
The basic reaction mixture contained: potassium phosphate, 50 mm, pH 7.8; indole, 0.5 mm; indoleglycerolphosphate, 0.5 mm (specific radioactivity, 400 nCi per pmole); and 0.0259 units of tryptophan synthase, in a final volume of 5.0 ml. Additions: pyridoxal phosphate, 0.2 mm; l-serine, 40 mm. Reaction mixtures were incubated at 37°. Samples (1.0 ml) were removed at zero time and at 10-min intervals for 30 min. Rate of Reaction 2 was measured as indicated under “Methods.” Rate of Reaction 3f was determined by measuring the radioactivity which appeared in indole as the incubation proceeded. Rates of the reactions were linear over the period of the assay.

| Tube | Additions | Product | Reaction 2 | Reaction 3f |
|------|-----------|---------|------------|-------------|
| 1    | None      | pmoles/10 min | 0          | 0.0023      |
| 2    | Pyridoxal phosphate | pmoles/10 min | 0.00430    |
| 3    | L-Serine  | pmoles/10 min | 0.00333    |
| 4    | L-Serine + pyridoxal phosphate | pmoles/10 min | 0.311       | 0.00980     |

radioactivity appeared in indole. If neither pyridoxal phosphate nor serine were added (tube 1), tryptophan synthesis was not detected and no change in the concentrations of either indoleglycerolphosphate or indole was detected. However, exchange was detected at the low but easily measured rate of 2.63 pmoles per hour. It was of considerable interest and significance that this rate, not detectable by the usual colorimetric procedures, is almost exactly that expected since, as shown in Fig. 1, Reaction 3f proceeds at a rate essentially 100-fold lower than the rate of Reaction 2. Such an interpretation is supported by the observed stimulation of the rate of exchange by the single addition of pyridoxal phosphate (tube 2) and the absence of stimulation by the single addition of serine (tube 3). Three single additions have already been shown to elicit similar responses by Reaction 3r (2). The observed exchange provides further support for the existence of indole as an enzyme-bound intermediate resulting from reaction of indoleglycerolphosphate at Site I.

A somewhat more detailed analysis of the data presented in Fig. 2 provided further support for the concept of indole channelling by tryptophan synthase. For this purpose the rates of formation of tryptophan in each of the reaction mixtures were computed. These rates were linear during the 40-min period under examination. Linear regression analyses were performed for each set of data, and correlation coefficients in excess of 0.98 were obtained in all cases. Increments of tryptophan which occurred during 10 min of reaction were obtained directly from these curves. The concomitant rates of appearance of radioactivity in tryptophan (raw data supplied to data repository) were nonlinear and were best described by a general exponential equation of the form Y = a × e^{bX}. Nonlinear regression analyses were performed testing the goodness of fit to this equation for each set of data on appearance of radioactivity in tryptophan. Correlation coefficients in excess of 0.96 were obtained. Increments of radioactivity which occurred during 10 min of reaction were taken directly from these curves. The average specific radioactivity of indole entering Site II and subsequently entering tryptophan will be referred to as the specific radioactivity of tryptophan-precursor indole and can be estimated during a given 10-min period from the expression:

\[
\Delta \text{ nCi per ml per 10 min in tryptophan} = \frac{\Delta \text{ pmoles tryptophan per ml per 10 min}}{\text{Average specific radioactivity of tryptophan-precursor indole}}
\]

The results of these calculations are presented in Fig. 2 along with the measured values of specific radioactivity for indoleglycerolphosphate, indole in the solvent, and tryptophan. The following trends are apparent in these data.

1. The rate at which the specific radioactivity of tryptophan increased was in all cases inversely proportional to the initial concentration of indole present in the reaction mixture.
2. The disparity between the specific radioactivity of tryptophan-precursor indole and of free indole in the solvent decreased as the initial concentration of indole in the solvent increased.
3. No obvious decrease in the specific radioactivity of indoleglycerolphosphate was observed during the first 40 min of each reaction.

One can arrive at this assessment for a given free indole concentration from the following information: (a) measured specific radioactivity of indoleglycerolphosphate (curves connecting open circles, Fig. 2); (b) measured specific radioactivity of solvent indole (curves connecting open triangles, Fig. 2); (c) calculated specific activity of tryptophan-precursor indole (curves connecting X's); (d) observed increments in tryptophan (curves connecting closed squares, Fig. 2).

These values are substituted in the following pair of simultaneous equations. (a) Moles \( \text{InGP} \) × specific radioactivity of \( \text{InGP} \) + moles solvent indole × specific radioactivity solvent indole = moles tryptophan increment × calculated specific radioactivity of tryptophan precursor indole, and (b) \( \text{Ind}_{\text{solvent}} + \text{Ind}_{\text{solvent}} = \text{moles of tryptophan increment} \). Solution of these equations yields the molar quantities of indoleglycerolphosphate and indole in the solvent which have contributed indole moieties to the population of tryptophan precursors channelled in the microenvironment which are available for reaction at Site II during any specified 10-min period of a reaction. The ratio of these quantities, \( \nu \), is given by the expression:

\[
\nu = \frac{\text{Ind}_{\text{solvent}}}{\text{Ind}_{\text{solvent}}}
\]

where \( \text{Ind}_{\text{solvent}} \) = moles of indole contributed by solvent indole, \( \text{Ind}_{\text{solvent}} \) = moles of indole contributed by solvent indoleglycerolphosphate. This ratio is a convenient way of expressing the relative contribution of each species to tryptophan-precursor indole channelled in the microenvironment. The relationship of the value of \( \nu \) to the concentration of indole in the reaction mixture for the first 40 min of several of the reaction mixtures (B through K and G through J, Fig. 2, plus three other reactions supplied initially with higher levels of indole) is shown in Fig. 5. Three conclusions follow from this analysis.
The estimation of \( \rho \) is discussed in the text. As defined, \( \rho \) is the ratio of solvent indole to indole generated at Site I which is available at Site II for conversion to tryptophan.

1. The precursor indole arriving at Site II is not wholly derived from indoleglycerolphosphate nor from indole in the solvent.

2. The mixing ratio, as inferred from the specific radioactivity of the channeled precursor indole, is not a simple linear function of the concentrations of indoleglycerolphosphate and indole in the solvent.

3. Solvent indole, even at greater than saturating concentrations (with respect to Site II) does not completely exclude indole moieties derived from solvent indoleglycerolphosphate. Thus, channeled indole is used preferentially.

These conclusions are consistent with the concepts advanced by Davis (6) in his "surface model" and extend the model of DeMoss (1) to include an enzyme-indole complex. The possibility of such a complex has recently been suggested by Kirschner and Wiskocil (8).

Consequently, attention was focused on the problem of obtaining direct experimental evidence for the existence of an enzyme-indole complex. The experiments detailed in Table II were carried out in an effort to find indole intimately associated or exchangeably bound to the surface of the enzyme. For these experiments nanomole quantities of tryptophan synthase were supplied with nearly saturating levels of radioactive [2-\(^{14}\)C]indoleglycerolphosphate (specific radioactivity, 8.0 \( \mu \)Ci per \( \mu \)mole). No other substrates or cofactors were supplied. The mixture was incubated briefly at 37\(^\circ\). Under these conditions the enzyme will convert some of the indoleglycerolphosphate to indole.

After about 10 min the net conversion stops presumably for the reason that equilibrium of the reversible reaction (37) is achieved (1). Samples of the reaction mixture (4.0 ml) were then passed through an ultrafilter which retained molecules in excess of 50,000 molecular weight. The material retained by the ultrafilter was resuspended in 4.0 ml of 50 mM potassium phosphate buffer (pH 7.8) which was 0.5 mM in nonradioactive indole.

The resuspended material was incubated 30 min at 30\(^\circ\). The mixture was then passed through the ultrafilter again and indole present in the filtrate was examined for radioactivity. The radioactivity associated with indole in this second filtrate was 0.17% of the originally supplied indoleglycerolphosphate. In all cases the specific radioactivity of the indole moiety of the compounds present in these mixtures was 8.0 \( \mu \)Ci per \( \mu \)mole.

The experiments described here were carried out in a tryptophanless mutant (td 120) of \( N.\ crassa\). This mutant has been shown (9) to have no detectable protein catalytically related to tryptophan synthase. Crude extracts of this mutant were subjected to the same protocol of purification (2, 10) as that employed to prepare catalytically active enzyme. Protein of the appropriate fraction was purified to homogeneity.

### Table II

| Conditions | Indole associated with material retained by ultrafilter |
|------------|-------------------------------------------------------|
|            | Experiment 1a | Experiment 1b | Experiment 2a | Experiment 2b |
|            | Sample 1 | Sample 2 | Sample 1 | Sample 2 |
| Tryptophan synthase | 205 | 208 | 234 | 198 |
| Catalytically inactive protein | 111 | 110 | 165 | 150 |
| No protein | 109 | 111 | 129 | 131 |

* Samples of 4.0 ml of a mixture containing tryptophan synthase (3.74 units per ml; 2.5 mg per ml); inactive protein (2.5 mg per ml); or no protein.

* Samples of 4.0 ml of a mixture containing tryptophan synthase (3.40 units per ml; 2.1 mg per ml); inactive protein (2.9 mg per ml); or no protein.

### Notations

\( p \) is the ratio of solvent indole to indole generated at Site I which is available at Site II for conversion to tryptophan.

\( \rho \) is the ratio of solvent indole to indole generated at Site I which is available at Site II for conversion to tryptophan.

\( \mu \)Ci per \( \mu \)mole.

### References

1. Davis, L. (6) in his "surface model" and extend the model of DeMoss (1) to include an enzyme-indole complex. The possibility of such a complex has recently been suggested by Kirschner and Wiskocil (8).

2. Samples of 4.0 ml of a mixture containing tryptophan synthase (3.40 units per ml; 2.1 mg per ml); inactive protein (2.9 mg per ml); or no protein.

3. Mixture of Experiment 1 contained per ml: 136 nmoles of indole, 100 nmoles of tryptophan, and 141 nmoles of indoleglycerolphosphate. Mixture of Experiment 2 contained per ml: 158 nmoles of indole, 41 nmoles of tryptophan, and 260 nmoles of indoleglycerolphosphate.

4. Mixture of Experiment 1 contained per ml: 110 nmoles of indole, 2 nmoles of tryptophan, and 255 nmoles of indoleglycerolphosphate. Mixture of Experiment 2 contained per ml: 162 nmoles of indole, 7 nmoles of tryptophan, and 278 nmoles of indoleglycerolphosphate.

5. Mixture of Experiment 1 contained per ml: 121 nmoles of indole, <1 n mole of tryptophan, and 182 nmoles of indoleglycerolphosphate. Mixture of Experiment 2 contained per ml: 152 nmoles of indole, 3 nmoles of tryptophan, and 107 nmoles of indoleglycerolphosphate. In all cases the specific radioactivity of the indole moiety of the compounds present in these mixtures was 8.0 \( \mu \)Ci per \( \mu \)mole.

6. Samples of 4.0 ml of a mixture containing tryptophan synthase (3.74 units per ml; 2.5 mg per ml); inactive protein (2.5 mg per ml); or no protein.

7. Samples of 4.0 ml of a mixture containing tryptophan synthase (3.40 units per ml; 2.1 mg per ml); inactive protein (2.9 mg per ml); or no protein.

8. Mixture of Experiment 1 contained per ml: 136 nmoles of indole, 100 nmoles of tryptophan, and 141 nmoles of indoleglycerolphosphate. Mixture of Experiment 2 contained per ml: 158 nmoles of indole, 41 nmoles of tryptophan, and 260 nmoles of indoleglycerolphosphate.
active protein. In a separate set of controls, protein was omitted from the reaction mixture, and the protocol was repeated to obtain the filter background.

The results reported in Table II show that protein mixtures containing catalytically active tryptophan synthase have the ability to bind indole exchangeably to an extent considerably above that which can be ascribed either to background in the assay procedures or to a generalized or nonspecific binding of indole to protein.

In similar experiments not reported here, mixtures like those described above were passed over Sephadex G-25 columns (2.5 x 40 cm). In these experiments almost no radioactivity was detected in the excluded volume containing the protein and enzymic activity. These experiments did show a slight but reproducible skewing of the leading edge of the indole peak. Interpretation of these results is complicated by the observed affinity of the gel for indole. These results may indicate that the presence of an exchangeable species is not absolutely required for removal of radioactivity from the indole-enzyme complex.

**DISCUSSION**

**Indole as Intermediate**—Free indole is not detected by colorimetric methods during the course of Reaction 1 of tryptophan synthase (2). In the presence of labeled indoleglycerolphosphate and nonradioactive indole, the specific radioactivity of the tryptophan was determined. This fact coupled with the observation that Reaction 3f proceeds, as usually measured, at a rate about 1 2/0 that required to sustain the rate of over-all conversion of indoleglycerolphosphate to tryptophan led Yanolsky and Rachmeler (3) to conclude that indole is not an obligate intermediate in the conversion of indoleglycerolphosphate to tryptophan. DeMoss (1) re-examined this question by supplying the enzyme with tritium-labeled indoleglycerolphosphate, nonlabeled indole, and other substrates and cofactors necessary for tryptophan production. In such a reaction mixture, indole was utilized before indoleglycerolphosphate for tryptophan formation. The trypophan formed contained appreciable amounts of radioactivity. Indole, too, became radioactive.

The specific radioactivity of the tryptophan, however, was 4 fold higher than that of indole. DeMoss' interpretation was that small amounts of indoleglycerolphosphate were going to tryptophan without equilibration with free indole and that radioactivity which appeared in indole did so as a result of the reversibility of Reaction 3. Thus, he concluded, also, that free indole is not an intermediate in the conversion of indoleglycerolphosphate to tryptophan. The experiments described in this report suggest that indole may be regarded as an enzyme-bound or "channeled" intermediate in the conversion of indoleglycerolphosphate to tryptophan. This suggestion is supported by the following evidence:

1. As the concentration of indole in the solvent was increased, its ability to trap radioactivity passing from indoleglycerolphosphate to tryptophan increased (Fig. 4).
2. At concentrations of indole on the order of 2.5 mM, the compound is completely effective as a trapping pool (Fig. 4).
3. The demonstration of bound indole on preparations of enzyme separated from reaction mixtures in which Reaction 3f was occurring.
4. The demonstration that, when Reaction 1 was conducted in the presence of labeled indoleglycerolphosphate and nonlabeled indole, the specific radioactivity of tryptophan precursor available at Site II for condensation with activated serine was very different from either indoleglycerolphosphate or indole in the solvent.

This interpretation is consistent with those of Lue and Kaplan (5) and of Gaertner et al. (12) in that it invokes a channeled intermediate. In this case, however, the argument is applied to a single enzyme rather than to a supramolecular aggregate. The evolutionary implications of this interpretation are discussed more extensively below.

**Nature of Indole-Enzyme Complex**—That indole is exchangeably bound to the enzyme is indicated by the operations employed in the experiment of Table I. Simply resuspending the retained enzyme in a solution containing a large excess of nonradioactive indole was sufficient to convert the radioactivity to a form which would pass readily through the filter. It is possible to estimate the amount of indole thus associated with the enzyme, by making a few apparently reasonable assumptions. For Experiment 1 of Table II, each sample contained 14.9 units of enzyme. The data of Meyer et al. (10) indicate that Y. crassa tryptophan synthase has a molecular weight of 150,000 and a maximal specific activity of 3.34 units per mg. Thus, each sample contained 29.4 moles of enzyme. The indole associated with this amount of enzyme over and above that of the controls was 83.5 nmoles. Thus, 2.8 moles of indole were bound per mole of enzyme. For Experiment 2 similar calculations revealed 2.9 moles of indole per mole of enzyme. Experiments now in progress (see below) in this laboratory indicate that the Neurospora enzyme exists as a dimer composed of subunits of 75,000 molecular weight. Thus, it would appear that each subunit has the ability to bind at least a molecule of indole under the conditions of the experiment of Table II.

**Mechanism of Action**—A model that attempts to accommodate previous work and the evidence presented in this report is depicted in Fig. 6. The K_m for indoleglycerolphosphate in Reaction 1 is nearly the same as the K_m for indole in Reaction 2 but the K_m for indole in Reaction 3r is about 30-fold higher than either of these values (cf. Fig. 1). Thus, the affinity of Site II for indole is greater than that of Site I for indole. The V_max for Site I, as judged by Reaction 1, is about one-half that for Site II as judged by Reaction 2. Thus, the conditions for the "surface model" of Davis (6) are met. Indoleglycerolphosphate bound at Site I is converted to indole at a rate sufficient to saturate the potential maximal rate at which Site II can convert indole to tryptophan. In the case of Reaction 1, indole generated at Site I is transferred or channeled immediately to Site II in a manner that prevents its escape to the solvent. Data presented here indicate that the Site I to Site II transfer of indole is about 200-fold more probable than its escape to the solvent. The presence of indole in the solvent at low saturating concentrations with respect to Site II interrupts this process in a way that provides insight into the mechanism of the reaction. Solvent indole partially engages Site II thereby reducing the probability of transfer of indole from Site I. Indole in the process of being transferred (channeled) is apparently able to exchange with the solvent indole (cf. Fig. 3). The presence of indole in the solvent reduces considerably the rate at which net conversion of indoleglycerolphosphate to tryptophan can occur in the absence of solvent indole (cf. Fig. 2). This may be explained by assuming that the total availability of indole now exceeds the ability of Site II to convert it to tryptophan. Thus the "channel" would be filled to capacity causing a high local concentration of the bound intermediate in the vicinity of Site I and consequent reduced availability of space at the site for continued turnover of indoleglycerolphosphate. In the absence of indole in the solvent, Site II is able to remove indole from the channel at an apparent rate 2-fold higher than its rate.
of production by Site I. In this case the channel is effectively empty and Site I is maximally available for binding and turnover of indoleglycerolphosphate.

The apparent inability of Site I and therefore Reaction 3f to produce indole at a rate sufficient to accommodate the observed rate of Reaction 1 may be accounted for in the same terms. Reaction 3f may be detected only in the absence of serine. In the absence of serine Site II may bind indole but obviously could not produce tryptophan. Thus, Site II could not remove indole from the channel. Escape of indole to the solvent would then occur at a rate much lower than its transfer to Site II and the absence of serine Site II may bind indole but obviously does not produce indoleglycerolphosphate.

The native complex, $\alpha\beta$, is required for catalysis of Reaction 1. Of particular interest is the fact that the maximal rates of Reactions 2 and 3 as catalyzed by the native complex are much higher than those observed with the individual subunits. The $\beta_2$ subunit alone will catalyze the deamination of serine but this activity is almost completely absent in the $\alpha\beta_2$ complex (cf. Ref. 13 and literature cited therein). Arrenhius and Wiskocil (8) in studies of steady state kinetics and fast reaction studies on the $E. coli$ enzyme have suggested that in the native configuration ($\alpha\beta_2$) more efficient conformations of the active sites are either induced or stabilized.

In the *Neurospora* system it seems unnecessary to invoke this level of conformational flexibility. Studies presently in progress (in collaboration with J. A. DeMoss) on electrophoretically homogeneous preparations of the *Neurospora* enzyme indicate a molecular weight of 150,000. The relatively mild conditions which cause the *E. coli* enzyme to dissociate apparently do not affect the *Neurospora* enzyme. The *Neurospora* enzyme is devoid of serine-deaminating activity (2, 10). Treatment of the enzyme with sodium dodecyl sulfate (2% sodium dodecyl sulfate at 100°C for 15 min followed by 30 min at 37°C) and polyacrylamide gel electrophoresis by the procedure of Weber and Osborn (14) reveals the presence of a single band with a relative mobility suggesting a molecular weight of 75,000. These data would indicate that the *Neurospora* enzyme is a dimer consisting of two subunits, each a single polypeptide analogous to an $\alpha$ chain covalently linked to a $\beta$ chain. Experiments are now in progress to test this assertion critically.

The selective advantages and evolutionary significance of multienzyme complexes and channeling of intermediates have been pointed out by Davis (6). The evidence for channeling of indole in tryptophan synthase of *Neurospora* presented in this report may provide some insight regarding evolution of the enzyme. Bonner's theory (15) of the evolution of gene-enzyme systems is based in large part on several features of the tryptophan synthases from several organisms. It states that non-identical catalytically active polypeptide chains which interact to form complex enzymic activities (e.g. the $\alpha$ and $\beta$ chains of the *E. coli* system) may in the course of evolution be converted into a single polypeptide under the control of a single genetic unit (e.g. the *Neurospora* system). Bonner pointed out a selective advantage that a single gene-single enzyme system would have over a multicomponent system. Once such a multicomponent system became established, coordination of the rate of formation of each of the components would be required to avoid wasteful synthesis of one of them. The most effective means of coordination would be to combine the involved genetic loci into a single unit coding for a single polypeptide. Another advantage accruing to such a unified system might be reduction or elimination of the conformational flexibility which is apparently a characteristic of interacting components (8, 13). Thus the catalytically active sites of the unified system would be
constrained in an efficient conformation and positioned so as to minimize escape or “unchanneling” of transient intermediates. In this context, the Neurospora enzyme may be interpreted as a unified system evolved from a two-component segment of the primitive pathway retaining most, but not all, of the catalytic activities of its precursors and a highly effective means of channeling indole, the apparent primitive penultimate intermediate in the biosynthesis of tryptophan.

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