BBA 65809

TRYPTOPHAN SYNTHASE FROM *NICOTIANA TABACUM**

DEBORAH P. DELMER** AND STANLEY E. MILLS
Department of Biology, University of California, San Diego, La Jolla, Calif. (U.S.A.)

(Received June 17th, 1968)

SUMMARY

A study has been made of the properties of a tryptophan synthase (L-serine hydro-lyase (adding indole), EC 4.2.1.20) obtained from cultured cells of *Nicotiana tabacum* var. Wisconsin 38. The data presented show that the enzyme more closely resembles the two-component bacterial type of tryptophan synthase in contrast to the single-component fungal enzyme.

1. K_m values for the substrates indoleglycerol phosphate, indole, L-serine, and pyridoxal phosphate are 0.11 mM, 0.016 mM, 34.0 mM, and 1.3 μM, respectively. The pH optimum for Reaction 2 activity (indole + L-serine → L-tryptophan) of the enzyme is 8.2.

2. The enzyme can be separated into two components by differential precipitation with $(NH_4)_2SO_4$. Component B alone catalyzes the conversion of indole to tryptophan and this activity is not stimulated by the addition of Component A. The combination of Components A and B is necessary for the conversion of indoleglycerol phosphate to tryptophan. Component A is heat-labile with a sedimentation constant of 3.1 S. Component B is heat-stable with a sedimentation constant of 5.5 S.

3. Mixed complementation experiments with the A and B subunits of *Escherichia coli* and *N. tabacum* tryptophan synthase, as well as antibody neutralization experiments, indicate that the B protein has undergone less change than the A protein throughout the course of evolution.

INTRODUCTION

In recent years, studies on the biosynthesis of tryptophan in a variety of microorganisms have revealed a number of diversities in the types of genetic control and aggregation of enzyme subunits of the pathway[1,2]. The first of such diversities to be recognized concerned the terminal enzyme, tryptophan synthase (L-serine hydro-lyase (adding indole), EC 4.2.1.20)[3]. In all organisms studied, this enzyme catalyzes 3 reactions[4-6].

* A preliminary report of this work appeared as an abstract in *Federation Proc.*, 27 (1968) 3342.

** Present address: Department of Chemistry, University of Colorado, Boulder, Colo. 80301 (U.S.A.).

1. Indoleglycerol phosphate + L-serine $\xrightarrow{\text{pyridoxal phosphate}}$ L-tryptophan + D-glyceraldehyde 3-phosphate

2. Indole + L-serine $\xrightarrow{\text{pyridoxal phosphate}}$ L-tryptophan

3. Indoleglycerol phosphate → indole + D-glyceraldehyde 3-phosphate

In *Escherichia coli* the enzyme can be separated into two components, the A and B proteins[4]. Component A alone can, to a limited extent, catalyze Reaction 3 and B alone, also to a limited extent, can catalyze Reaction 2. Both reactions are, however, markedly stimulated by the formation of an A–B complex. The A–B complex is necessary for the catalysis of Reaction 1. Reaction 1 is apparently the physiological reaction and does not involve indole as a free intermediate[5,6]. From genetic evidence it is known that the A and B proteins are coded by two cistrons which map adjacent to each other[7].

All bacteria examined as well as the blue-green alga *Anabena viriabilis* and green alga *Chlorella ellipsoidea* (K. SAKAGUCHI, personal communication) possess the two-component enzyme. On the other hand, both genetic and biochemical data for the enzyme in the fungus *Neurospora crassa* indicate that the A and B genes may have fused to form a single gene which codes for a protein that behaves as a single component upon purification[8,9]. Similarly, in another ascomycete *Aspergillus nidulans*[10] and in the yeast *Saccharomyces cerevisiae* (T. MANNEY, personal communication), genetic evidence indicates that a single gene codes for tryptophan synthase. From the standpoint of biochemical evolution it was therefore of interest to us to examine the enzyme in a more highly-evolved organism of the plant kingdom.

There is some evidence in the literature for the presence of a tryptophan synthase in plants. GREENBERG AND GALSTON[11] have reported in crude extracts of pea seedlings a tryptophan synthase activity with a pH optimum of 7.8. A recent report by CHEN AND BOLL[12] confirmed this activity in pea seedlings but assigned a pH optimum of 8.4 to the enzyme. NAIR AND VAIDYANATHAN[13] have partially purified an enzyme with such activity from the seeds of the Bengal gram. This enzyme is different in one respect since it possesses a pH optimum of 5.5. All groups measured only one of the 3 reactions catalyzed by the microbial enzyme, the condensation of indole and serine to form tryptophan; none characterized the subunit structure of the enzyme.

We have been studying the biosynthesis of tryptophan in cells of *Nicotiana tabacum* var. Wisconsin 38 cultured *in vitro*[14]. The data presented in this paper concerning a tryptophan synthase obtained from callus tissue of *N. tabacum* indicate that the general properties of the enzyme closely resemble those of all the microbial tryptophan synthases studied, and that the tobacco enzyme possesses the character of an easily-separable two-component enzyme of the bacterial and algal type.

MATERIALS AND METHODS

Growth of tissue

Callus tissue from *Nicotiana tabacum* var. Wisconsin 38 has been continually subcultured for 4 years in our laboratory on the amino acid-free solid medium described previously[14]. Cells were harvested for assays between days 8 and 14 after subculturing and enzyme activity remained fairly constant over this time interval.

Contamination controls

Because of the low levels of activity observed, it was necessary to eliminate contamination as a possible source of enzyme activity. Based on the following reasons, we believe we have eliminated contamination as a source of activity: (a) Cells were never harvested for enzyme assays from flasks which were visibly contaminated. (b) Cell homogenates prepared aseptically yielded no microbial growth when plated on complex bacterial and fungal media. (c) Homogenates prepared by breakage in a glass tissue homogenizer and centrifuged at speeds which pellet bacteria yielded crude extracts containing tryptophan synthase of specific activity comparable to that obtained in the freeze-dry method described below. (d) On first attempts, enzyme activity was demonstrated in young seedlings as well as older leaves of *N. tabacum* plants, in callus tissue of the hybrid of *Nicotiana glauca* × *Nicotiana langsdorfii*, and in cultured cells of *Haplopappus gracilis*.

Enzyme preparation

Callus tissue taken directly from agar medium was frozen in liquid N_2 and lyophilized to dryness. Cells dried in this manner could be stored at −70° for at least 1 month with no loss of activity. To prepare crude extracts, the dried tissue (equivalent to 20 g fresh weight of tissue) was ground with Polyclar-AT, then extracted at 4° with 20 ml 0.2 M potassium phosphate (pH 8.5) containing 20 μg/ml pyridoxal phosphate and 5.0 mM mercaptoethanol. The resulting suspension was centrifuged at 4° at 18 000 rev./min for 5 min. The pellet was re-extracted with 5 ml of the same buffer and centrifuged as before. The supernatants were pooled and designated as crude extract. For optimum Reaction 1 activity in crude extracts, the molarity of the potassium phosphate buffer was lowered to 0.05 M.

Separation of A and B components

All procedures were performed in the cold. 0.1 vol. of 1.5% protamine sulfate was added dropwise to a crude extract. The solution was stirred continually for 5 min, centrifuged at 18 000 rev./min for 10 min and the pellet discarded. Powdered enzyme-grade $(NH_4)_2SO_4$ was then added to the supernatant with constant stirring to bring the solution to 35% satn. The solution was stirred for 10 min, centrifuged at 18 000 rev./min for 5 min and the pellet resuspended in 0.1 vol. of buffer. This preparation contained all of the Reaction 2 activity. Small amounts of residual A activity could be removed by heating the preparation at 80° for 1 min. The 35% supernatant was brought to 70% satn. with $(NH_4)_2SO_4$. After stirring for 10 min, the suspension was centrifuged at 18 000 rev./min for 5 min and the pellet was resuspended in 0.1 vol. of buffer. This fraction contained the majority of the A activity and was devoid of B activity. When the A and B fractions were to be mixed and assayed for Reaction 1 activity, they were individually desalted by passage through a Sephadex G-25 column equilibrated in 0.05 M potassium phosphate (pH 8.5), pyridoxal phosphate at 20 μg/ml, and mercaptoethanol at 1.0 mM.

Assays

All reactions were linear at 37° for at least 1 h. To assay Reaction 1, 0.1 μmole of [^{3}H]indoleglycerol phosphate (specific activity of indole moiety was 2830 counts/min per mμmole), 40 μg of pyridoxal phosphate, 40 μmoles of L-serine, and 50 μmoles of

potassium phosphate (pH 8.5) were incubated with enzyme to a final volume of 1.0 ml. The reaction was incubated for 1 h at 37°, then terminated by placing the tubes in a boiling-water bath. The reaction was then extracted twice with 4.0 ml toluene, and the layers were separated by centrifugation. 0.7 ml of the aqueous layer was assayed for tryptophan by the addition of a 20-fold excess of a partially-purified preparation of tryptophanase prepared from a strain of *E. coli* which was devoid of tryptophan synthase activity. Tryptophanase catalyzes the conversion of L-tryptophan to indole *plus* pyruvate and NH_3. The indole released was extracted into toluene and an aliquot of the toluene layer placed in 10 ml toluene scintillation fluid and counted in a Nuclear-Chicago liquid scintillation counter. One unit of enzyme activity was defined as one mμmole tryptophan produced per h.

To assay Reaction 2, the same procedure was followed with the substitution of 0.2 μmole [^{3}H]indole (specific activity 1100 counts/min per mμmole) in place of [^{3}H]indoleglycerol phosphate, and the quantity of potassium phosphate (pH 8.5) was increased to 200 μmoles.

Protein was assayed by the method of LOWRY *et al.*[15].

Sucrose gradient centrifugation was performed by the general method of MARTIN AND AMES[16]. Desalted, concentrated enzyme (0.3 ml) was layered onto a 5–20% sucrose gradient made up in 0.1 M potassium phosphate (pH 8.0) *plus* 20 μg/ml pyridoxal phosphate and 1.0 mM mercaptoethanol. The gradients were centrifuged for 11 h at 4° in an SW-65 swinging bucket rotor at 62 000 rev./min in a Beckman Model L-2 ultracentrifuge. Hemoglobin was used as a marker for *s*-value comparison. 10-drop fractions were collected for assays. To detect A component on the gradient by means of Reaction 1, B component was added to all fractions before assaying. No activity for Reaction 1 was ever detected on a gradient without the addition of B component to the assay mix.

Antibody neutralization experiments

Antiserum against purified *E. coli* A protein was the generous gift of Dr. TERENCE MURPHY. Antisera against *E. coli* B protein and *Neurospora crassa* tryptophan synthase were obtained from rabbits injected with purified B protein and partially purified *Neurospora crassa* tryptophan synthase, respectively. The experiments were performed with *N. tabacum* A and B protein, separated and concentrated by $(NH_4)_2SO_4$ precipitation. Enzyme was pre-incubated with antiserum for 5 min at 37° prior to the addition of substrate. Neutralization of Reaction 2 activity by either anti-*E. coli* B serum or anti-*Neurospora crassa* serum was measured both in the absence and presence of *Nicotiana tabacum* A protein and both results were identical (< 2% variation). The data presented for Reaction 2 neutralization were performed in the presence of *Nicotiana tabacum* A protein.

Chemicals

Uniformly ^{3}H-labeled anthranilic acid was prepared by the method of WEGMAN AND DEMOSS[17]. [^{3}H]indoleglycerol phosphate was prepared enzymatically from [^{3}H]-anthranilic acid by the following procedure: 70 μmoles of [^{3}H]anthranilate, 76 μmoles phosphoribosyl pyrophosphate, 30 μmoles $MgSO_4$, 300 μmoles potassium phosphate (pH 7.0), 116 units (1 unit = 1 μmole product formed/h) of partially-purified *Neurospora crassa* phosphoribosyl transferase and 160 units of *Neurospora crassa* partially-

purified enzyme aggregate possessing both isomerase and indoleglycerol phosphate synthase activity were combined in a final volume of 29.0 ml. The reaction was incubated for 2 h at 37° and was terminated by placing the reaction in a boiling-water bath for 3 min. Denatured protein was removed by centrifugation. The supernatant, containing [^{3}H]indoleglycerol phosphate, was extracted extensively with toluene to remove radioactivity which was toluene-extractable. The specific activity of the [^{3}H]indoleglycerol phosphate was determined by its conversion to [^{3}H]indole with purified *E. coli* A protein. The indole formed was extracted into toluene. An aliquot of the toluene layer was placed in 10 ml toluene scintillation fluid and counted in a Nuclear Chicago liquid scintillation counter. Molar amount of indole counted was determined by assaying another aliquot for indole by the color reaction described by YANOFSKY[18].

Uniformly ^{3}H-labeled indole was prepared enzymatically from L-[^{3}H]tryptophan by the method of MATCHETT AND DEMOSS[19].

Uniformly ^{3}H-labeled L-tryptophan was purchased from International Chemical and Nuclear Corporation. Phosphoribosyl pyrophosphate was purchased from P.L. Biochemical Labs; L-serine and pyridoxal phosphate were obtained from Cal-Biochem. Polyclar-AT powder was purchased from General Aniline and Film Corporation. Protamine sulfate was purchased from Elanco Products Co. Phosphoribosyl transferase, isomerase, and indoleglycerol phosphate synthase were the generous gifts of Dr. J. A. DEMOSS. Purified *E. coli* A protein was a gift of Dr. TERENCE MURPHY; *E. coli* B protein was purified by the method of WILSON AND CRAWFORD[20].

RESULTS

Initial attempts to demonstrate tryptophan synthase activity in crude extracts prepared from callus tissue of *N. tabacum* were unsuccessful. For this early work the conventional assay was used which measures the disappearance of indole in the presence of L-serine and pyridoxal phosphate. Crude extracts were assayed under varying conditions of pH. Some indole disappearance was detected at pH 5–6, but it was not serine-dependent and no newly-synthesized tryptophan was detected. However, when the sensitivity of the assay was increased by the use of radioactive indole, it was possible to detect both indole disappearance and tryptophan production, although not in a stoichiometric relationship. Examination of this point convinced us that to obtain valid results, it was necessary to measure tryptophan production rather than indole disappearance. This conclusion was based on data which showed that the kinetics of tryptophan production were linear, reproducible, and serine-dependent. Such was not the case with indole disappearance.

General properties of the enzyme

Figs. 1 and 2 describe some of the general properties of the enzyme. K_m determinations for the substrates are shown in Figs. 1A–D. These determinations are summarized in Table I along with the K_m values reported for the *E. coli* and *Neurospora crassa* enzyme. The similarity to the microbial enzymes is apparent. The pH optimum of 8.2 for Reaction 2 (Fig. 2) is similar to that reported for the microbial tryptophan synthases and differs markedly from that of pH 5.5 reported for the tryptophan synthase from seeds of the Bengal gram[13].

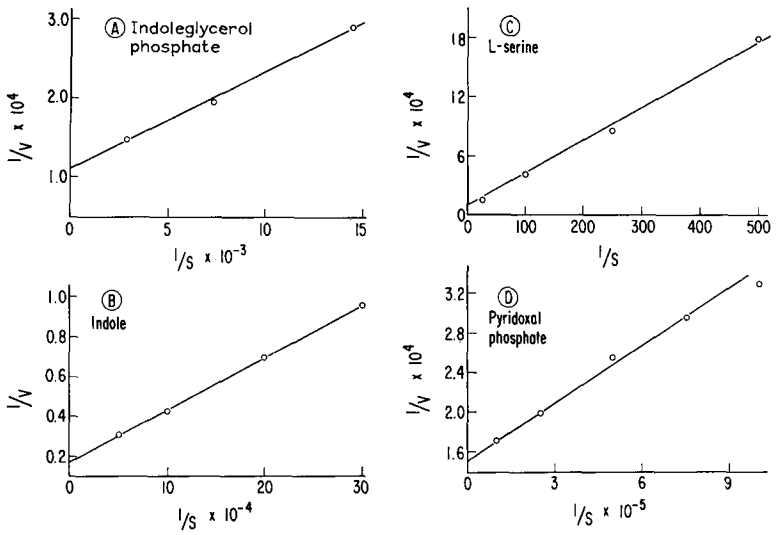


Fig. 1. K_m determinations for substrates. Assays were performed with enzyme concentrated by $(NH_4)_2SO_4$ precipitation and desalted with Sephadex G-25. The time interval (45 min) was chosen so that the reaction rate was linear at the substrate concentration used. K_m's for indole, L-serine, and pyridoxal phosphate were measured for Reaction 2. K_m for indoleglycerol phosphate was measured for Reaction 1. [S] = molarity. For 1A, 1C, and 1D, v = counts/min per h of L-[^{3}H]-tryptophan produced. For 1B, v = mμmoles/h of tryptophan produced.

It should be pointed out at this time, that only Reaction 1 (indoleglycerol phosphate + L-serine → tryptophan) and Reaction 2 (indole + L-serine → tryptophan) have been measured for the *N. tabacum* enzyme. Although an extensive variety of conditions has been employed (including extended periods of incubation in the presence and absence of B component and the use of agents which trap the reaction products), under no circumstances have we been able to detect Reaction 3 activity. Whether this activity alone does not exist for the plant enzyme or whether it is simply below the sensitivity of our assay cannot be determined. However, it can be calculated that, if the reaction exists, it must proceed at a rate less than 0.5% of Reaction 1 activity.

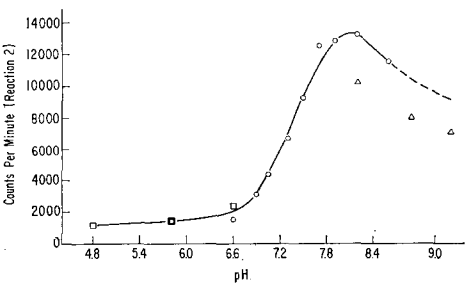


Fig. 2. pH optimum of the *N. tabacum* enzyme. Assays were performed on crude extracts. □ = acetate buffer; ○ = potassium phosphate buffer; △ = Tris buffer. Because of the stimulatory effect of salt on Reaction 2 activity, KCl was added to each reaction to adjust the K^+ concn. to 0.4 M. All buffers tested in the range pH 8.0–9.5 were inhibitory; thus, the shape of the curve (dotted line) was drawn to match the Tris data but extrapolated to activity comparable to that in potassium phosphate buffer.

TABLE I

SUMMARY OF K_m VALUES FOR VARIOUS TRYPTOPHAN SYNTHASES

The numbers in parentheses after the K_m values indicate the reaction measured for the determination.

Reference	*Substrate*	*Organism*		
		Escherichia coli	*Neurospora crassa*	*Nicotiana tabacum*
24, 6	Indole	0.165 mM (2)	0.07 mM (2)	0.016 mM (2)
6*	Indoleglycerol phosphate	0.15 mM (3)	0.20 mM (1)	0.11 mM (1)
24, 25	L-serine	24.6 mM (2)	4.8 mM (2)	34.0 mM (2)
24, 25	Pyridoxal phosphate	1.1 μM (2)	1.0 μM (2)	1.3 μM (2)

* And T. MURPHY, personal communication.

Subunit character of the enzyme

The results of the first experiment designed to determine the subunit character of the enzyme are presented in Table II. Following a protamine sulfate treatment, it was observed that essentially all Reaction 2 activity was precipitated by $(NH_4)_2SO_4$ added to 35% satn., but that the major portion of Reaction 1 activity was lost. This activity could be restored by the addition of an inactive fraction which precipitated between 35 and 70% satn. It appeared that differential precipitation with $(NH_4)_2SO_4$ caused the separation of the enzyme into two components analogous to the A and B proteins of the bacterial tryptophan synthases. Thus, the component precipitated between 35 and 70% satn. could be considered analogous to the A protein of *E. coli* which, only in combination with B, is capable of catalyzing Reaction 1.

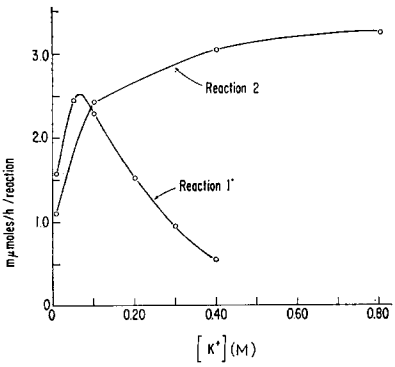
The ratio of rates for Reaction 2 to Reaction 1 for microbial tryptophan synthases is of the order of 1 to 2. This differs markedly from the ratio of 12 observed for these activities in *N. tabacum* crude extracts (Table II). Therefore, it seemed possible that Reaction 1 was not being measured under optimum conditions. In investigating this possibility it became evident that salt concentration had a profound and differential effect on the rates of Reactions 1 and 2. As can be seen from the data in Fig. 3, the rate of Reaction 2 increased sharply when the concentration of KCl was raised to

TABLE II

DISTRIBUTION OF ENZYME ACTIVITIES FOLLOWING $(NH_4)_2SO_4$ FRACTIONATION

Assays were carried out as described in MATERIALS AND METHODS. Reaction 1: Indoleglycerol phosphate + L-serine → L-tryptophan; Reaction 2: indole + L-serine → L-tryptophan.

Fraction assayed	*Counts/min tryptophan per h per reaction*		*mμmoles tryptophan per h per reaction*		*Ratio Reaction 2/ Reaction 1*
Reaction:	*2*	*1*	*2*	*1*	
Crude extract	15 750	4 230	14.30	1.20	11.9
0–35% ppt.	35 500	1 415	32.20	0.40	80.5
35–70% ppt.	200	0	0.18	0.0	
0–35% ppt. *plus* 35–70% ppt.	30 700	10 600	27.90	3.00	9.0



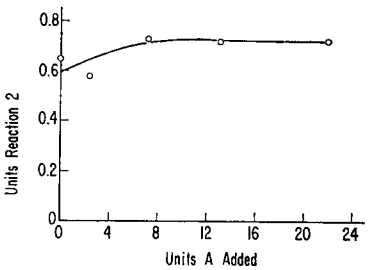


Fig. 3. The effect of salt concentration on Reactions 1 and 2. Enzyme was prepared from $(NH_4)_2SO_4$ precipitates desalted with Sephadex G-25 equilibrated in 0.01 M potassium phosphate (pH 8.5). Increasing salt concentration was obtained by the addition of KCl.

Fig. 4. The effect of component A upon component B Reaction 2 activity. Units Reaction 2 = mμmoles tryptophan formed from indole/h. Units of A = mμmoles formed from indoleglycerol phosphate/h in the presence of saturating B component.

0.1 M and more gradually up to 0.8 M. The rate of Reaction 1, however, was at a maximum between 0.05 and 0.1 M KCl and decreased rapidly at lower and higher salt concentrations. The ratio of 12 observed for the rates of Reaction 2 to Reaction 1 were initially measured at 0.3 M KCl, a salt concentration that was highly inhibitory to Reaction 1 while affording nearly maximum stimulation of Reaction 2. Subsequent work has shown that Reaction 1 is also inhibited by high concentrations of NH_4Cl and NaCl to about the same extent as with KCl.

A study of the effect of the A protein upon Reaction 2 (see Fig. 4) has shown that like the Anabena and Chlorella enzymes (K. SAKAGUCHI, personal communication), but unlike the *E. coli* enzyme, the catalysis of Reaction 2 by *N. tabacum* B protein is not stimulated by component A.

Studies on the heat stability of the A and B proteins yielded results similar to those obtained with the bacterial tryptophan synthases. The heat inactivation curves of Fig. 5 show that the A protein of the *N. tabacum* enzyme is relatively heat labile, losing 100% of its activity in 1 min at 80° and 50% of its activity in 3 min at 60°. The high heat stability of the B component is also characteristic of other tryptophan synthases.

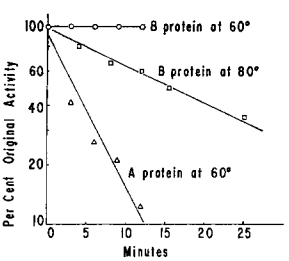


Fig. 5. Heat stability of *N. tabacum* A and B proteins. Heat inactivation was carried out on enzyme prepared by $(NH_4)_2SO_4$ and resuspended in 0.1 M potassium phosphate (pH 8.5) *plus* pyridoxalphosphate at 40 μg/ml. Following heat treatment, enzyme was cooled rapidly by placing tubes in an ice bath; assays were then performed as described in MATERIALS AND METHODS.

s-Values of the subunits; evidence for an A–B complex; complementation

The sedimentation behavior of the two components during sucrose-gradient centrifugation is shown in Fig. 6. Once again, the similarity to the bacterial enzyme is apparent in the size of the components; 3.1 S and 5.5 S for the *N. tabacum* A and B components, respectively, *versus* 2.7 S and 5.1 S for the *E. coli* A and B proteins[21]. We have been unable to demonstrate an A–B complex by this procedure. The S values for both components remain the same within experimental error whether A and B were layered on separate gradients or in combination on one gradient. The same result was obtained under varying conditions of serine, pyridoxal phosphate, or buffer concentration, including that salt concentration optimal for Reaction 1 activity. Thus, it appears that if A and B do form a complex, it is easily dissociable during sedimentation. It is possible, of course, that no A–B complex at all is formed for the catalysis of Reaction 1 and that this reaction simply represents the sequential catalysis of Reactions 3 and 2 carried out by the individual A and B proteins.

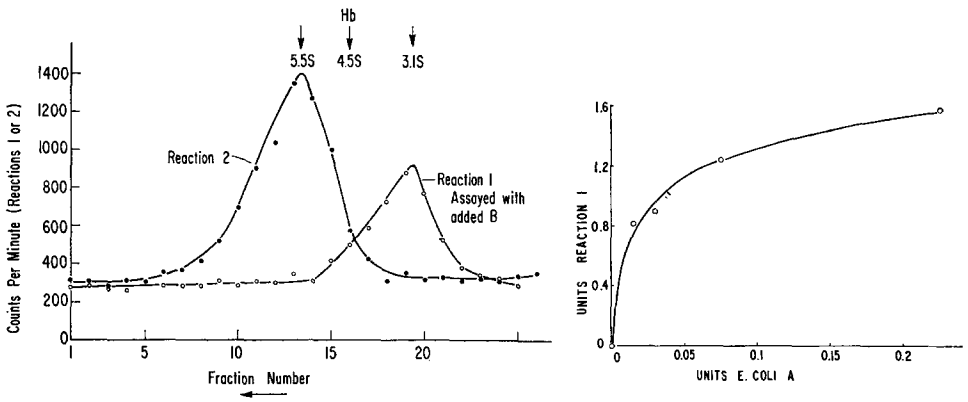


Fig. 6. Sucrose-gradient sedimentation pattern of *N. tabacum* A and B proteins. 0.3 ml of a mixture of concentrated A and B preparations was layered onto a 5–20% sucrose gradient containing 0.1 M potassium phosphate (pH 8.0) *plus* pyridoxal phosphate at 40 μg/ml. The gradient was centrifuged for 11 h at 4° at 62 000 rev./min.

Fig. 7. Complementation of *N. tabacum* B protein with *E. coli* A protein. Units = mμmoles tryptophan produced/h. A constant amount of *N. tabacum* B protein (that which yields 8 units of Reaction 1 activity in the presence of saturating *N. tabacum* A protein) was added to each reaction. The units of *E. coli* A refer to the Reaction 3 activity of the enzyme measured in the absence of B protein.

This assertion seems unlikely for the following reasons. First, as mentioned above, catalysis of Reaction 3 by the *N. tabacum* enzyme was not demonstrable under a variety of conditions. Second, the catalysis of Reaction 1 was not observed when the A protein of *N. tabacum* was mixed with an excess of either of two enzymes, each of which alone could catalyze Reaction 2. No Reaction 1 activity was obtained with a mixture of *N. tabacum* A and an extract of td141, a tryptophan-requiring strain of *Neurospora crassa* with a mutant tryptophan synthase capable of catalyzing only Reaction 2. Identical results were obtained with *N. tabacum* A and B protein of *E. coli*. These results clearly show that the catalysis of Reaction 1 by the plant enzyme cannot be described simply as the sequential catalysis of Reactions 3 and 2.

While there was no enzyme complementation between *N. tabacum* A and *E. Coli* B protein, when the reverse experiment was performed—the combination of purified *E. coli* A protein with *N. tabacum* B protein—it was observed that the catalysis of Reaction 1 did occur. The data for this experiment are presented in Fig. 7. As might be expected, the heterologous complex was less efficient since the final level of activity extrapolated to saturation was about 4-fold less than that obtained when the same amount of B is saturated with *N. tabacum* A protein. It seems reasonable to suggest that an actual A–B complex is being formed since the units of unstimulated *E. coli* A Reaction 3 activity added to these reactions is less than 10-fold the A–B activity obtained in the mixed reactions, indicating that the *N. tabacum* B protein is stimulating the *E. coli* A activity. Thus, it appears that the *N. tabacum* and the *E. coli* B proteins, in contrast to the A proteins, have retained enough similarities throughout the course of evolution to allow complementation to occur.

Antiserum neutralization experiments

In addition to the enzyme complementation results, further support for the conclusion that structural similarities have been preserved between the B protein of *E. coli* and *N. tabacum* came from antibody neutralization experiments. In this analysis

TABLE III

ANTISERUM NEUTRALIZATION EXPERIMENTS

N. tabacum A and B proteins, separated and concentrated by $(NH_4)_2SO_4$ precipitation were preincubated with antiserum for 5 min at 37° prior to the addition of substrate. 1 unit = 1 mμmole tryptophan produced per h. The same unit was used for the antiserum neutralization unit. Percent cross reaction (column 7) $= \frac{\text{column 5}}{\text{column 3}} \times 100$.

Reaction measured	*Antiserum added*	*Total neutralizing units added**	*N. tabacum enzyme activity units*	*Units neutralized*	*Percent cross reaction*
Experiment 1					
1	None	0	1.92	—	—
1	Normal serum	0	1.84	—	—
1	Anti-*E. coli*—A	4400	2.27	−0.35	—
1	Anti-*E. coli*—B	1660	0.02	+1.90	0.11
1	Anti-*N. crassa* tryptophan synthase	374	0.30	+1.62	0.43
Experiment 2					
2	None	0	12.65	—	—
2	Normal serum	0	12.70	—	—
2	Anti-*E. coli*—A	4400	15.30	−2.65	—
2	Anti-*E. coli*—B	1660	1.43	+11.22	0.68
2	Anti-*N. crassa* tryptophan synthase	374	4.45	+10.10	2.70
Experiment 3					
2	None	0	7.15	—	—
2	Anti-*E. coli*—apo B	123	2.38	+4.77	3.88

* The amount of antibody added is expressed as neutralizing units and refers to the ability of a specified quantity of antiserum to neutralize the activity of its homologous antigen. Thus, 4400 units of anti-*E. coli* A serum represents that quantity of serum which neutralizes 4400 units of *E. coli* A enzyme activity.

we used three rabbit antisera prepared against the following antigens; purified A and B proteins from *E. coli* and purified apo-B protein in which the pyridoxal phosphate cofactor of the *E. coli* B protein was removed by extensive dialysis of the protein against 10 mM DL-serine in Tris buffer. The data presented in Table III show that antiserum prepared against purified *E. coli* A protein is totally ineffective in neutralizing the *N. tabacum* enzyme activity. (In fact we obtain a reproducible stimulation of activity with this antiserum.) In contrast, both the anti-*E. coli* B protein and anti-apoenzyme antisera show a small but significant amount of neutralization of the plant enzyme. The results not only suggest the presence of one or more similar determinants in the two B proteins but also show that at least one of the shared determinants does not include the pyridoxal phosphate cofactor common to the two proteins. Interestingly enough, neutralization is also obtained with antiserum to a partially-purified preparation of tryptophan synthase obtained from *Neurospora crassa*. The cross-reaction obtained with the anti-*N. crassa* antiserum indicates that the 2-component plant enzyme shares at least one determinant with the single-component fungal enzyme.

Regulation

In contrast to the results with the tryptophan synthases of microbial origin, we have been unable to demonstrate any form of control over the activity or production of the enzyme in *N. tabacum* callus tissue. Growth on extreme excesses of L-tryptophan over a period of 4 generations where free tryptophan pools are elevated as much as 140-fold caused no measurable decrease in the specific activity of tryptophan synthase. Attempts to derepress the level of this enzyme by growth on a variety of tryptophan analogues, a successful technique for derepression in bacteria, similarly caused no change in the specific activity of the enzyme in crude extracts. Furthermore, of 9 indole analogues tested (including the plant hormone indoleacetic acid), only one, indoleacrylic acid, inhibited activity of the enzyme *in vitro*; the end product, L-tryptophan, caused no inhibition of *in vitro* activity when assayed in concentrations as high as 1 mM.

DISCUSSION

A comparison of the properties of a tryptophan synthase isolated from cultured cells of *N. tabacum* with the properties of the enzymes from other sources has revealed a number of interesting points. The notable difference between the pH optimum of the *N. tabacum* enzyme and that reported by NAIR AND VAIDYANATHAN[13] for the tryptophan synthase isolated from seeds of the Bengal gram suggests that these may be distinct enzymes. Furthermore, NAIR AND VAIDYANATHAN also reported that the Bengal gram enzyme was inhibited by indole proprionic acid; we have been unable to detect any inhibition of the *N. tabacum* enzyme by this compound. Because the *N. tabacum* enzyme isolated from callus tissue has been shown to catalyze Reaction 1, (the physiological reaction in microorganisms), and also because its other properties so closely resemble those of the physiological enzyme in microorganisms, we suggest that this represents the physiological tryptophan synthase in *N. tabacum*. Furthermore, we have recently demonstrated activity for the enzyme in young seedlings and in leaves of older *N. tabacum* plants. It would be of interest to determine whether the enzyme in the Bengal gram is a distinct tryptophan synthase, or whether it possesses

unique properties due to its existence in the seeds rather than the vegetative structures of the plant. It is also possible that the pH-5.5 enzyme performs a physiological role in the plant other than that of tryptophan biosynthesis.

Of further interest is the discovery of the conservation of a 2-component enzyme in a higher plant species. This observation serves to support the notion of the uniqueness of certain biochemical trends in the evolution of the fungi. In addition to the unique single-component tryptophan synthase, the fungi also possess distinct pathways for lysine[22] and niacin[23] biosynthesis. With respect to all of these characteristics, the higher plants follow the bacterial biochemical patterns and remain distinct from the fungi.

Perhaps the most interesting result obtained has been the demonstration of the degree of homology which still exists between the tryptophan synthase of a higher plant and a simple bacterium, particularly with regard to the B protein. The ability of the *N. tabacum* B protein to complement with the A protein of *E. coli* in the catalysis of a complex enzymatic reaction is an indication of how little the B protein of tryptophan synthase has altered in the course of evolution. It may be that the significant change which has evolved between the bacterial and higher plant mode of tryptophan biosynthesis has not occurred in the structure of the tryptophan synthase itself, but in the loss of any demonstrable form of control over the levels of activity of this enzyme *in vivo*.

ACKNOWLEDGEMENTS

This work was supported by Grant GB-3073 from the National Science Foundation and Contract No. AT(11-1)-34 from the Atomic Energy Commission. D.P.D. is an NIH Predoctoral Fellow. Fellowship No. 5-F1-GM-24,046-04.

REFERENCES

1 J. A. DEMOSS, *Biochem. Biophys. Res. Commun.*, 18 (1965) 850.
2 R. HÜTTER AND J. A. DEMOSS, *J. Bacteriol.*, 94 (1967) 1896.
3 D. BONNER, J. A. DEMOSS AND S. E. MILLS in V. BRYSON AND H. J. VOGEL, *Evolving Genes and Proteins*, Academic Press, New York, 1965, p. 305.
4 I. P. CRAWFORD AND C. YANOFSKY, *Proc. Natl. Acad. Sci. U.S.*, 44 (1958) 1161.
5 C. YANOFSKY AND M. RACHMELER, *Biochim. Biophys. Acta*, 28 (1958) 640.
6 J. A. DEMOSS, *Biochim. Biophys. Acta*, 62 (1962) 279.
7 C. YANOFSKY AND I. P. CRAWFORD, *Proc. Natl. Acad. Sci. U.S.*, 45 (1959) 1016.
8 D. BONNER, Y. SUYAMA AND J. A. DEMOSS, *Federation Proc.*, 19 (1960) 926.
9 M. CARSIOTIS, E. APPELLA, P. PROVOST, J. GERMERHAUSEN AND S. R. SUSKIND, *Biochem. Biophys. Res. Commun.*, 18 (1965) 877.
10 R. HÜTTER AND J. A. DEMOSS, *Genetics*, 55 (1967) 241.
11 J. B. GREENBERG AND A. W. GALSTON, *Plant Physiol.*, 34 (1959) 489.
12 J. CHEN AND W. G. BOLL, *Proceedings of the Canadian Society of Plant Physiologists, Ottawa, Ontario*, 8 (1967) 28.
13 P. M. NAIR AND C. S. VAIDYANATHAN, *Arch. Biochem. Biophys.*, 104 (1964) 405.
14 D. P. DELMER AND S. E. MILLS, *Plant Physiol.*, 43 (1968) 81.
15 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
16 R. G. MARTIN AND B. N. AMES, *J. Biol. Chem.*, 236 (1961) 1372.
17 J. WEGMAN AND J. A. DEMOSS, *J. Biol. Chem.*, 240 (1965) 3781.
18 C. YANOFSKY, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 2, Academic Press, New York, 1955, p. 233.
19 W. H. MATCHETT AND J. A. DEMOSS, *Biochim. Biophys. Acta*, 86 (1964) 91.

20 D. A. WILSON AND I. P. CRAWFORD, *J. Biol. Chem.*, 240 (1965) 4801.
21 T. E. CREIGHTON AND C. YANOFSKY, *J. Biol. Chem.*, 241 (1966) 980.
22 H. F. VOGEL in V. BRYSON AND H. J. VOGEL, *Evolving Genes and Proteins*, Academic Press, New York, 1965, p. 279.
23 C. YANOFSKY in W. D. MCELROY AND B. GLASS, *Amino Acid Metabolism*, The Johns Hopkins Press, Baltimore, 1955, p. 930.
24 M. HATANAKA, E. A. WHITE, K. HORIBATA AND I. P. CRAWFORD, *Arch. Biochem. Biophys.*, 97 (1962) 596.
25 J. A. DEMOSS AND D. BONNER, *Proc. Natl. Acad. Sci. U.S.*, 45 (1959) 1405.