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THE SIMILARITY OF TRYPTOPHAN SYNTHETASES OF *ANABAENA VARIABILIS* AND *CHLORELLA ELLIPSOIDEA* WITH THAT OF BACTERIA

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SUMMARY

1. Tryptophan synthetase (L-serine hydro-lyase, EC 4.2.1.20) has been demonstrated in extracts from pure cultures of *Anabaena variabilis*, a blue-green alga, and *Chlorella ellipsoidea*, a green alga. The characteristics of these enzymes were compared with that of bacteria and *Neurospora crassa*.

2. Both the algal tryptophan synthetases were separated on a Sephadex G-100 column into two components. One component (B) possessed full activity to form tryptophan from indole without the second component (A). A only in combination with B, was able to form tryptophan from indole glycerophosphate. Component A of the tryptophan synthetase from either *Escherichia coli* or *Salmonella typhimurium* could complement component B from either of the two algae in the conversion of indole glycerophosphate, but the algal component A could not complement bacterial component B.

3. Antiserum against Salmonella component B weakly neutralized Anabaena tryptophan synthetase, while antiserum against component B from Neurospora did not. On the contrary, Chlorella tryptophan synthetase was very weakly neutralized by antiserum to the Neurospora enzyme, but it was not affected by Salmonella antiserum.

4. Phylogenetically discussed, the two algal enzymes were considered to belong in the bacterial group rather than in the fungal group.

INTRODUCTION

Tryptophan synthetase (L-serine hydro-lyase, EC 4.2.1.20) has been studied in a fungus *Neurospora crassa*[1] and in various bacteria such as *Escherichia coli*[2], *Salmonella typhimurium*, *Serratia marcescens*[3,4] and *Bacillus subtilis*[5]. In every case it has been shown to catalyze three reactions.

Indole glycerophosphate + L-serine → L-tryptophan (1)
Indole + L-serine → L-tryptophan (2)
Indole glycerophosphate → indole + D-glyceraldehyde 3-phosphate (3)

In *N. crassa* all 3 reactions are catalyzed by a single protein species[6,7].

The enzyme from another fungus, *Aspergillus niger*, showed a cross-reaction with antibody against *N. crassa* td. 141 enzyme but not with antibody against Salmonella enzyme (KENJI SAKAGUCHI, unpublished results). The same phenomenon was observed in the case of Saccharomyces, another Ascomycetes[8].

The tryptophan synthetase of the above four bacterial species was composed of two dissociable protein components termed A and B. Investigations with *E. coli*, *S. typhimurium* and *S. marcescens* have shown that the two proteins are required for the maximum activity of the above three reactions, and that component A (mol. wt. about 30 000)[9] possesses the active center for Reaction 3, whereas component B (mol. wt. about 100 000)[10] is the site for Reaction 2. The physiological reaction (Reaction 1) depends on the integrity of both components and does not involve free indole. The enzyme from *B. subtilis* was similar in this respect, but its component B effected Reaction 2 without component A.

The structural similarity of the enzyme from *E. coli*, Salmonella and Serratia was tested by two methods[11]. In combining the two components from the three enteric bacteria, no decrease of complementation efficiency was observed on Reaction 2, while the antiserum against *E. coli* component A showed a difference of neutralizing efficiency compared to the other component A derived from Salmonella and Serratia (30% and less than 5%, respectively). Antiserum against *E. coli* component B neutralized the B component from the other two species of enteric bacteria with equal efficiency, but precipitation in gel by the OUCHTERLONY[24] technique showed spurs and disclosed differences among them.

The peptide pattern of α-subunits (A-proteins) disclosed common peptide regions between *E. coli*, *S. typhimurium* and *Aerococcus aerogenes*, and nearly identical patterns within *E. coli* species and *Shigella dysenteriae*[12,13].

Tryptophan synthetases were also demonstrated in higher green plants[14-16]. However, their dissociability was not studied, their relationship with bacterial or fungal enzyme being unclarified.

From the genealogical point of view, *A. variabilis*, a blue-green alga, and *C. ellipsoidea*, a representative green alga, were selected and investigated. Both algae contain a tryptophan synthetase dissociable into two components rather similar to the bacterial type. Their properties and correlation with fungal or bacterial enzymes are also described.

MATERIALS AND METHODS

Culture of A. variabilis

A pure strain of *A. variabilis* (Meyer's strain) was received from Dr. Francis Haxo's laboratory. Cells were grown in Kratz-Meyer's inorganic medium[17]. Air mixed with 0.5–3% CO_2 was sterilized through a cotton filter and bubbled into the medium. The cultures were irradiated with light from two banks of fluorescent lamps to 50 ft-candles. Cultures in 40-l carboy flasks were carried out at 23° for 2 weeks, harvested using a Sharples centrifuge and washed with 0.1 M phosphate buffer (pH 7.8). The packed cells were frozen and stored at −60°. No loss of enzyme activity was observed during 2 months.

Enzyme assays

The activity of the three reactions was assayed by the method of DEMOSS[18]. The microassay method was used to detect the enzyme activity for Reactions 1 and 2 (ref. 5). When the enzymic activity was too low to be detected by colorimetric method, 0.1 μmole of [^{14}C]indole was used in the reaction mixture, and the radioactivity of the [^{14}C]tryptophan produced was counted[19]. Indole glycerophosphate was prepared enzymically from anthranilic acid by a procedure developed by SMITH AND YANOFSKY[20]. Protein was estimated by the method of LOWRY *et al.*[21]. The specific activity of the enzyme is defined as the number of μmoles of tryptophan formed, per the number of μmoles of substrate utilized, per mg protein per h.

Partial purification of Anabaena tryptophan synthetase

All operations were performed at 0–6°. All centrifugations were carried out in a refrigerated centrifuge at 13 000 × *g* for 20 min or 30 000 × *g* for 15 min. Thawed cells, suspended in 0.1 M Tris citrate buffer (pH 7.8) were disrupted in a French press. After centrifugation to remove cell debris, 1.5% protamine sulfate solution (pH 7.0) was slowly added to a volume 1/7 or 1/6 of the supernatant, of which the protein concentration was around 10 mg/ml. After stirring for 20 min the solution was centrifuged. The supernatant was supplemented with NaCl to a final concentration of 1.5 M, DL-serine to $1 \cdot 10^{-4}$ M, pyridoxal phosphate to $4 \cdot 10^{-5}$ M, EDTA to $2 \cdot 10^{-4}$ M, and GSH to $2 \cdot 10^{-4}$ M, in order to stabilize the enzyme against heat treatment. The pH was adjusted to 7.8, the solution was heated to 55° in a 90° water bath and immediately transferred to a water bath of 55° where the temperature was held at 55° for 5 min. After immersion in ice, the solution was centrifuged. The protein precipitated from the supernatant between 20 and 65% $(NH_4)_2SO_4$ saturation was collected. This precipitate could be stored in the frozen state if necessary.

The precipitate was dissolved in a buffer of Tris citrate (0.05 M at pH 7.8), supplemented with NaCl (1.5 M), pyridoxal phosphate ($2 \cdot 10^{-4}$ M) and GSH ($1 \cdot 10^{-4}$ M). Column chromatography was carried out on a 4.5 cm × 100 cm column of Sephadex G-100 which had been equilibrated with the above mentioned buffer at 4°. 5-ml fractions were collected approximately every 5 min. The elution of protein was followed by absorbance measurement at 280 mμ. Component B fractions were estimated by the microassay method for Reaction 2, the appropriate fractions heat-treated at 80° for 10 min, and the precipitate discarded after centrifuging. Usually, a 60-fold purified Anabaena component B was obtained.

Culture of C. ellipsoidea

200 ml of a pure culture of *C. ellipsoidea* (Tamiya's strain) were received from Dr. S. Miyachi in Dr. A. Benson's laboratory. 50 ml of the culture were inoculated into 4 l of Miyachi's medium in 5-l conical flasks, light being irradiated from the bottom and both sides to an intensity of about 200 ft-candles. In the early period of growth, the light intensity was diminished.

The culture medium (Miyachi's medium) contained KNO_3 (1.26 g/l), $MgSO_4 \cdot 7H_2O$ (0.63 g/l), KH_2PO_4 (0.31 g/l), $FeSO_4$ (0.006 g/l) and Kratz Meyer's microelement solution 1 ml/l (ref. 10). Air mixed with 3% CO_2 was sterilized through a cotton filter and bubbled into the medium.

Cells were harvested after culturing for about 5 days. After washing once, the packed cells were frozen and stored at −60°.

Partial purification of Chlorella tryptophan synthetase

The procedures were identical to those used to purify the tryptophan synthetase of Anabaena, except that the heat treatment was omitted since the Chlorella enzyme is heat labile. An extract of the cells was prepared by disrupting the thawed cells suspended in 0.1 M Tris citrate buffer (pH 7.8) by passing the cell suspension 3 times through a French press. After centrifugation and protamine sulfate treatment to remove cell debris and nucleic acid, the protein precipitating from this supernatant at 25–70% $(NH_4)_2SO_4$ saturation was collected. The precipitate was dissolved in a buffer containing 1.5 M NaCl in the same manner as described for the purification of the Anabaena enzyme and dispensed onto the top of a Sephadex G-100 column. Fractions containing the components B and A were found by the microassay method for Reactions 2 and 1.

Materials

Indole glycerophosphate was prepared according to the method of DEMOSS[18]. [^{14}C]Indole was purchased from the International Chemical and Nuclear Corp., City of Industry, Calif. Neurospora tryptophan synthetase antiserum was a generous gift from Drs. S. Ensign and S. Kaplan. Serratia tryptophan synthetase component A and the antibody against Salmonella component B were supplied through the courtesy of Dr. E. Balbinder.

TABLE I

THREE REACTIONS OF ANABAENA TRYPTOPHAN SYNTHETASE

To assay Reaction 1, 50 μmoles of potassium phosphate buffer (pH 7.8), 20 μg of pyridoxal phosphate, 40 μmoles of L-serine and 0.5 μmole of indole glycerophosphate were incubated with the enzyme preparation in a final volume of 1.0 ml at 37°, and tryptophan was assayed as described below. To assay Reaction 2, the same procedure was utilized except that 0.5 μmole of indole was substituted for the indole glycerophosphate, and the disappearance of indole was determined as described below. To assay Reaction 3r, 50 μmoles of potassium phosphate buffer (pH 7.8), 6.6 μmoles of indole and 6.6 μmoles of glyceraldehyde phosphate were incubated with the enzyme preparation, and indole glycerophosphate was determined as described below. In certain cases 20 μg of pyridoxal phosphate and 40 μmoles of L-serine were added to the reaction mixture. The reactions, after the incubation at 37° for 30 min., were stopped by heating in boiling water for 2 min and ice-cooling. The reaction mixture was then extracted with 4.0 ml of toluene, and an aliquot of the toluene layer was assayed for indole by acidified *p*-dimethylaminobenzaldehyde reagent. The reaction mixture was extracted again, and tryptophan was determined by the tryptophanase procedure[18]. Indole glycerophosphate remaining in the aqueous layer was assayed by the periodate oxidation procedure[18].

Reaction	*Enzyme preparation*	*Activity*
1 Indole + serine → tryptophan	Crude extract	0.011 μmole/h per mg protein
2 Indole + serine → tryptophan	Crude extract	0.027 μmole/h per mg protein
3r Indole + glyceraldehyde phosphate → indole glycerophosphate	Mixture of partially purified A and B components	0.021 μmole/ml per h

RESULTS

Tryptophan synthetase of A. variabilis

Three reactions of Anabaena enzyme. The crude extracts showed a low activity for Reactions 1 and 2 (Table I), the specific activity being about one-tenth that of the *N. crassa* crude extract. The rate of Reaction 1 did not exceed that of Reaction 2, suggesting the possibility of a two-component system. Since Reaction 3 and the reverse reaction were very slow even when the reaction mixture was supplemented with high concentrations of indole and glyceraldehyde phosphate to accelerate the reverse reaction[18], partly purified components A and B, which were fractionated on a Sephadex G-100 column, were mixed, and their activity on the reversed Reaction 3 was demonstrated (Table I, Fig. 1).

Chromatography. A typical pattern of protein and enzyme activities obtained by chromatography is shown in Fig. 1. The component B activity was observed (Tubes 20–27) without supplementation of component A. The recovery of Reaction 2 activity was over 80%. None of the fractions of component B were able to convert indole glycerophosphate into tryptophan.

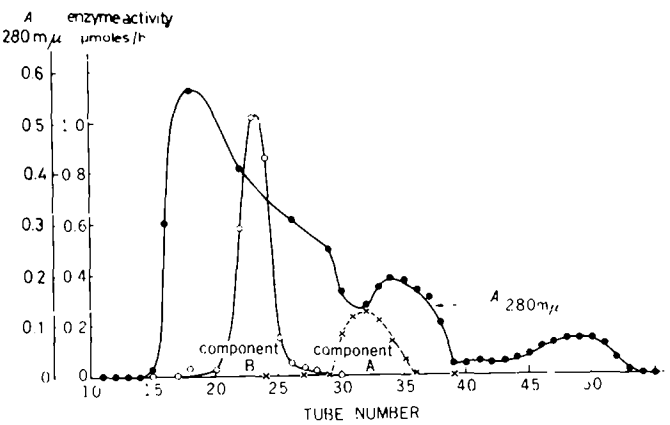

Fig. 1. Separation of Anabaena tryptophan synthetase into two components with Sephadex G-100 column. Fractionation procedure was as described in MATERIALS AND METHODS. Component B was assayed for Reaction 2, and component A was assayed in the presence of the saturating amount of component B for Reaction 1 as described in the legend of Table I. Eluting buffer: 0.05 M Tris citrate (pH 7.8), 1.5 M NaCl, $1 \cdot 10^{-3}$ M EDTA, $4.0 \cdot 10^{-5}$ M pyridoxal phosphate, $1 \cdot 10^{-4}$ M glutathione.

The distribution of component A activity in Fig. 1 could be detected only by supplementation of component B. Component A itself showed no reaction, or very slight activity in converting indole and glyceraldehyde phosphate to indole glycerophosphate.

Whether Anabaena component B is completely independent for Reaction 2 or whether component A can contribute to the reaction to any extent was tested, since component B of members of Enterobacteriaceae have low activity for Reaction 2, which is greatly enhanced by the addition of component A (refs. 3, 4). The results in Table II indicate that Anabaena component B was fully active in forming tryptophan from indole without any addition of component A.

TABLE II

INDEPENDENCE OF ANABAENA COMPONENT B FOR THE REACTION OF INDOLE + SERINE → TRYPTOPHAN ACTIVITY

The assay procedure was as described in the legend of Table I. Activity is expressed as μmole/h per 0.1 ml.

Component	*Indole → tryptophan*	*Indole glycerophosphate → tryptophan*
A	0.00	0.00
B	0.285	0.00
A* + B	0.290	0.180

* A saturating amount of component A was added to component B.

The doubt that component A may have contaminated the component B fraction was excluded in this experiment by heating the component B fraction to 80° for 10 min in 1.5 M NaCl solution. Only component B could survive this heat treatment and component A was completely destroyed, as will be described later. Table II also shows that the physiological reaction (indole glycerophosphate → tryptophan) was only catalyzed through cooperation of the two components, neither component having any activity in this reaction by itself. It is noteworthy that SCHWARTZ AND BONNER[5] reported the independent activity of *B. subtilis* component B fraction in Reaction 2, though qualitatively[4].

Effect of various salts on the activity of Reaction 2. The effect of K_2HPO_4, KCl, NaCl, NH_4Cl and sodium citrate on the Anabaena B component was tested. Each salt was added to the reaction mixture at a concentration of 0.1-2 M, incubated for 20 min at 37°, and the disappearance of indole assayed. In the same way, the tryptophan synthetase from *N. crassa* td 141 (an indole-utilizing mutant) was tested for its stimulation by salt concentration of 2 M, and only about a 20% decrease was observed with K_2HPO_4 and NH_4Cl at concentrations above 1 M.

The Anabaena enzyme showed a slight increase of its activity at 1 and 2 M NaCl and sodium citrate. However, this effect is far below the cationic effect on *E. coli* and *B. subtilis* tryptophan synthetase, for which about a 3-fold increase of Reaction 2 activity was shown with 1 M NH_4Cl and KCl, respectively[5,22]. Neurospora enzyme did not show any increase in activity with addition of salts. In this respect, Anabaena enzyme did not resemble the enterobacterial enzyme.

Effect of NaCl on the heat stability of component B. The increase of Reaction 2 activity by NaCl suggests a change in the thermostability of component B with salt concentration. Component B of *A. variabilis*, which has a rather high optimal growth temperature of 37°, was stable on heat treatment at 70° for 10 min in 0.05 M Tris buffer (pH 7.8). The inclusion of 1.5 M NaCl enhanced the heat stability of component B against heating at 80° for 30 min and also its tolerance to freezing and thawing. This enhanced stability was an advantage for the purification of this enzyme.

On the other hand, component A of this algal enzyme was completely destroyed by heating at 70° for 10 min, even with the addition of 1.5 M NaCl.

Thermal inactivation curve of component B. Thermal inactivation of component B was examined in an attempt to confirm that component A was not required for Reaction 2. *E. coli* and Salmonella component B could express only about one-hun-

dredth of their full activity without component A. If Anabaena component B required its component A for full expression of its activity, a two-component thermal inactivation curve should have been observed. It is to be remembered that under the test conditions, the algal component A was far more labile than component B, and the algal enzyme showed only a small increase in its activity by the supplementation of NaCl. Fig. 2 shows that the heat-stable component in the enzyme solution was less than 2%, and this was probably caused by the visible agglutination of the protein following heating at 90°. From this curve, and from the results obtained by chromato-

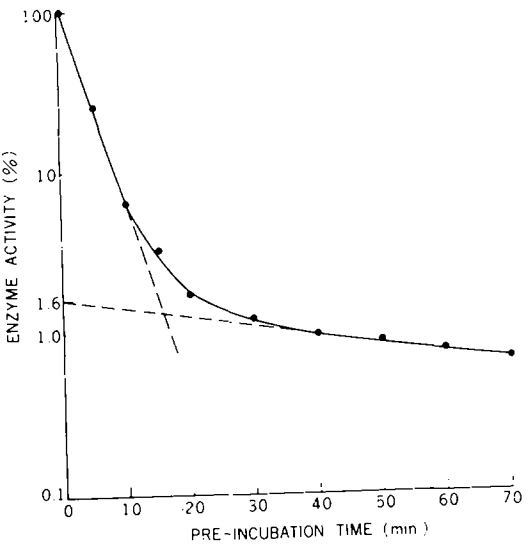

Fig. 2. Heat inactivation of Anabaena tryptophan synthetase component B. The component was preincubated at 90° for the indicated period, cooled in ice, and the remaining enzyme activity for Reaction 2 was assayed as usual. Reaction: indole + serine → tryptophan. Condition: 90° in 0.05 M Tris–citrate buffer (pH 7.8), 1.5 M NaCl, $1 \cdot 10^{-3}$ M EDTA, $4 \cdot 10^{-5}$ M pyridoxal phosphate, $1 \cdot 10^{-5}$ M glutathione.

graphic separation of the two components, it was concluded that component B of the Anabaena enzyme was independent from component A in Reaction 2, in forming tryptophan from indole.

Complementation of Anabaena tryptophan synthetase components with bacterial components. Since we have components A and B of Anabaena enzyme, it is possible to compare their relative affinities with bacterial component B or A by saturation experiments. In complementing a limited amount of Anabaena component B with various amounts of Salmonella component A, a saturation curve in Reaction 1 was obtained as presented in Fig. 3. 0.1 unit of Salmonella component A manifested only 0.007 unit of Reaction 1 activity when it was mixed with 0.1 unit of Anabaena component B.

The low affinity of the bacterial and algal components was demonstrated in another way. In mixing 1 unit of Salmonella component B with 0.1 unit of Anabaena component A, the enzyme activity reached saturation and stayed at 53% of the activity expressed by the normal algal components. This ratio of complementation

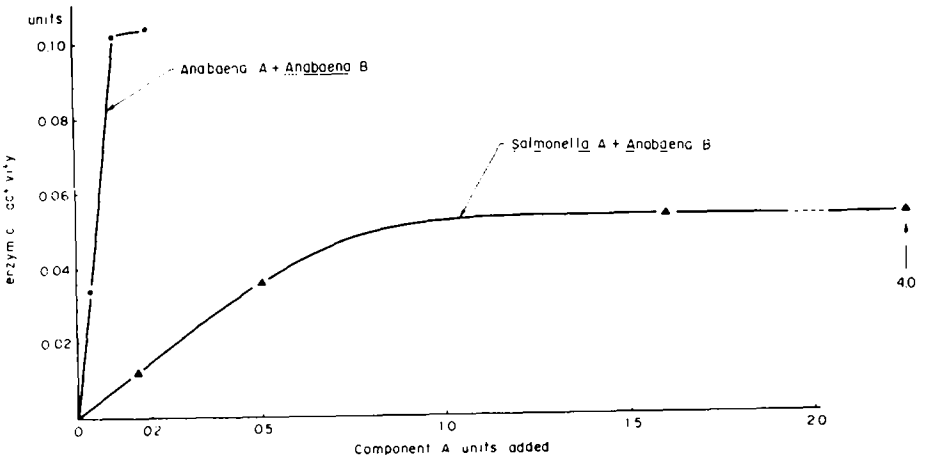

Fig. 3. Saturation curve of Anabaena component B with Anabaena and Salmonella component A. 0.1 unit of Anabaena component B was supplemented with various amounts of Anabaena or Salmonella component A, and its activity for Reaction 1 was assayed.

can be an index of relative affinity between two tryptophan synthetases from different sources (Fig. 3).

This ratio was assayed by combining Anabaena component A or B with over a 20-fold excess of Salmonella, *E. coli* component B or A, and with *S. marcescens* component A. The three bacteria are members of the Enterobacteriaceae, and in Reaction 1, Anabaena component B complemented the three bacterial A components, but their complementation ratios after saturation were all about 50%, showing similarity between the algal and bacterial B component. On the other hand, these data suggest the similarity of the A components of the three enteric bacteria mentioned above, since their complementation ratios were almost the same (Table III).

The algal component A was far more unrelated to the bacterial A components. This minor half of the Anabaena tryptophan synthetase did not show any activity in forming tryptophan from indole glycerophosphate when mixed with a large excess of bacterial B components (Table III). Although this phenomenon does not require

TABLE III

COMPLEMENTATION OF ANABAENA COMPONENT A OR B WITH SALMONELLA, *E. coli*, SERRATIA COMPONENT B OR A

Reaction 1 activity was compared, taking the combination of Anabaena components A and B as 100%.

	Anabaena		*Salmonella*		*E. coli*		*Serratia*
	A	*B*	*A*	*B*	*A*	*B*	*A*
Anabaena A		100		0		0	
Anabaena B	100		53		51		47

TABLE IV

INHIBITION OF ANABAENA TRYPTOPHAN SYNTHETASE ACTIVITY WITH SALMONELLA AND NEUROSPORA TRYPTOPHAN SYNTHETASE ANTISERUM

The Anabaena components were mixed with each serum, kept in ice for 10 min and then assayed for Reaction 2.

Enzyme	*Anabaena component B*			*Anabaena components A + B*	
*Antiserum (units)**	*Not added*	*Salmonella anti-B (5.5)*	*Neurospora antiserum (20)*	*Not added*	*Neurospora antiserum (20)*
Enzyme activity (unit) for Reaction 2	0.44	0.28	0.44	0.44	0.44
Neutralization** efficiency (%)		2.9	0		0

* 1 unit antiserum = the amount of antiserum which neutralizes 1 unit of homologous enzyme.

** Neutralization efficiency (%) = (unit of enzyme activity inhibited)/(unit of antiserum added) × 100.

a complete difference between the two kinds of A-protein, at least some large difference in their conformation and amino acid sequence is suggested.

Immunological difference between bacterial and fungal tryptophan synthetase. In order to compare the general structural similarity of the two kinds of protein, the immunological method was applied. The cross-reaction between Anabaena enzyme and antiserum against Neurospora enzyme was tested by precipitation in agar gel in a PREER[23] tube and by the OUCHTERLONY[24] method. The enzyme solution used was a crude extract of Anabaena cells. Its enzyme had a specific activity of 0.44 unit. The antiserum against the purified enzyme from *N. crassa* td 141 was a gift from Dr. S. Kaplan[6,25]. No cross-reaction was observed between the Anabaena enzyme and the fungal antiserum by these techniques.

Neutralization of the algal enzyme with the bacterial antiserum was also not observed, as shown in Table IV. However, a weak but definite neutralization effect was manifested in applying the antiserum against Salmonella component B on Anabaena enzyme. The neutralization efficiency, which was defined as the percentage of a unit of enzyme activity inhibited per unit of antiserum added, was as low as 2.9%, while a neutralizing efficiency of 30% was observed in combining Salmonella component A with antiserum against *E. coli* component A. It is considered that this value can be an index of the similarity between two proteins obtained from different sources.

Tryptophan synthetase of C. ellipsoidea

The crude extract of *C. ellipsoidea*, prepared as described in MATERIALS AND METHODS, elicited the three reactions of tryptophan synthetase (Table V). Its separation into two components was made by following the method applied to the Anabaena crude extract. The activity of component B was measured in the reaction forming tryptophan from indole, no increase in activity being observed by supplementing Chlorella or *E. coli* component A. On the other hand, the Chlorella component A was detected only in the presence of Chlorella or Anabaena component B in Reaction 1.

TABLE V

THREE REACTIONS OF *C. ellipsoidea* TRYPTOPHAN SYNTHETASE

Crude extract was used as the enzyme solution and assayed as described in the legend of Table I.

Reaction	*Activity (μmole/h per mg protein)*
1 Indole glycerophosphate + serine → tryptophan	0.020
2 Indole + serine → tryptophan	0.022
3r Indole + glyceraldehyde phosphate → indole glycerophosphate	0.007

No activity of the three reactions was observed using component A alone. An attempt to complement Chlorella component A with bacterial component B gave a negative result, as in the case of Anabaena component A. Fig. 4 shows that a good separation of the two components of Chlorella enzyme was obtained using the Sephadex G-100 column eluted with 1.5 M salted buffer. Component B was larger than component A, as is found with bacteria and Anabaena. It is noteworthy that the two algal enzymes had the same type, a two-component system, which was complemented by bacterial component A but not by bacterial component B, and characterized by the independence of component B in forming tryptophan from indole. Also, the two components of the two algal enzymes complemented each other efficiently, especially in combining with Anabaena component A, the Chlorella component B proceeding in Reaction 1 more effectively than its original A component.

Immunological tests. Immunochemical experiments were carried out on Chlorella enzyme using antisera against purified Neurospora td 141 enzyme and against Salmonella component B, following the method applied to Anabaena. In the neutral-

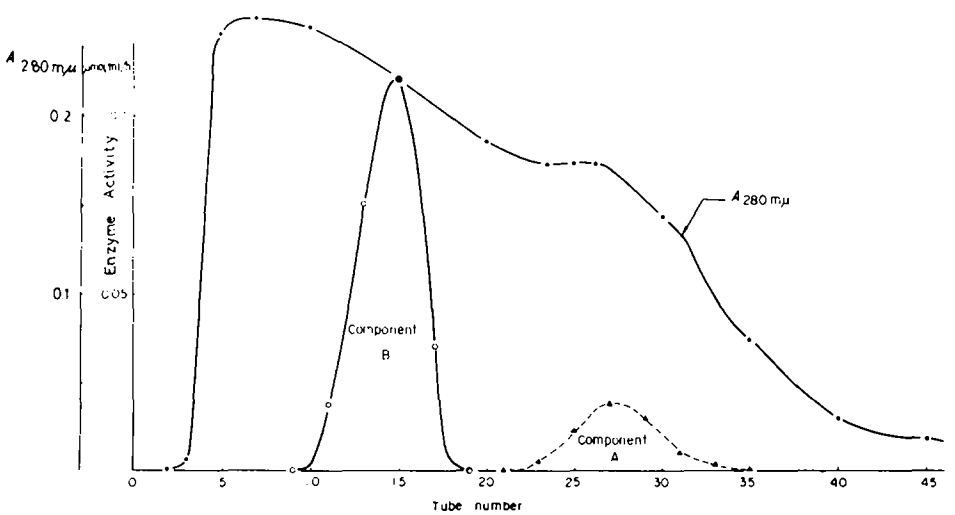

Fig. 4. Separation of Chlorella tryptophan synthetase into two components with Sephadex G-100 column. The procedures were identical to those described in Table I. Component A was assayed for Reaction 1 in the presence of a saturating amount of component B.

TABLE VI

PROPERTIES OF TRYPTOPHAN SYNTHETASE OBTAINED FROM NEUROSPORA, ANABAENA, CHLORELLA OR *E. coli*

	Number of components	*Stimulation by salt*	*Complementation with*									*Cross reaction with*		*Enhancement by component A on Reaction 2*
			Salmonella		*E. coli*		*Anabaena*		*Chlorella*		*Serratia*	*Neurospora anti serum*	*Salmonella B anti-serum*	
			A	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*			
Neurospora (td 141)	1				—							+	—	No
Anabaena	2		—		—	—	—	—			+	—	+ (2.9%)	No
			(53%)		(51%)		(100%)	(100%)			(47%)			
Chlorella	2	—	—		—	—				—	—	+ (0.05%)		No
E. coli	2				—				—			—		Yes

ization experiment, the enzyme activity was assayed using [^{14}C]indole as a substrate.

The precipitation reactions of crude extract of the Chlorella with the fungal and bacterial antisera were both negative when tested by the agar gel diffusion methods of OUCHTERLONY[21] and PREER[23]. However, a slight neutralizing effect was observed when the antiserum against Neurospora enzyme was applied to the Chlorella component B. Its neutralization efficiency against the units of the antiserum was less than 1%, but evidently the reaction was positive in duplicate experiments, one assayed by radioisotope counting and the other by colorimetric assay. In both cases, the antiserum against Salmonella enzyme and the neutral serum failed to inhibit the Chlorella enzyme. This may suggest a partial similarity of the molecular shape between Chlorella component B and *N. crassa* enzyme. All other experiments tested showed that *C. ellipsoidea*, a typical green alga, had the same bacterial type of tryptophan synthetase as the blue-green algae. Also, the two algal enzymes had much more similarity to each other than to the bacterial enzyme. The results in this report are summarized in Table VI.

DISCUSSION

Tryptophan synthetase is one of the enzymes with which the most elaborate biochemical, genetical and immunochemical investigations were carried out, both in fungi and bacteria, providing good foundation to use the tryptophan synthetase of different organisms as the evolutionary parameter from the biochemical stand point.

The two algae, *A. variabilis*, a blue-green alga, and *C. ellipsoidea*, a green alga, are in general, considered as intermediates between unicellular and nonphotosynthetic microorganisms and higher plants. The blue-green algae have especially been considered to have many morphological and taxonomical correlations with bacteria such as their mode of fission, size and shape and their cellular constitution. The green algae such as Chlorella has advanced cellular constitutions like grana, mitochondria and so on, and undergoes photosynthesis by chlorophyll.

The fact that both algal tryptophan synthetases have two-component systems and their B components can complement with A components of enterobacterial enzymes indicates that the tryptophan synthetase of blue-green and green algae are more similar to that of bacteria than that of fungi. The independence of the two algal B components to proceed in Reaction 2 indicates that they are more similar to *B. subtilis* tryptophan synthetase than enterobacterial enzymes.

The unexpected phenomenon that the Chlorella enzyme was weakly but significantly inhibited by Neurospora antiserum, but not by Salmonella antiserum, is really the only one divergence from the general relatedness of the two algal tryptophan synthetases from the bacterial type. One can accept this fact as a revealed token of the relationship of Chlorella enzyme with that of fungi, but it also can be considered as an accidental copy of an enzymatically active configuration of fungal tryptophan synthetase, which has occurred through many mutations during the process of evolution. In general, it is doubtless that *C. ellipsoidea* has an enzyme of bacterial tendency.

From these facts, one can postulate a map of evolutional correlation between bacterial, fungal and algal tryptophan synthetases, by following the scheme of BONNER *et al.*[4] with minor modifications (Fig. 5). The general principle of this map is that the kind of an enzyme within the related species is the same type. This principle has been

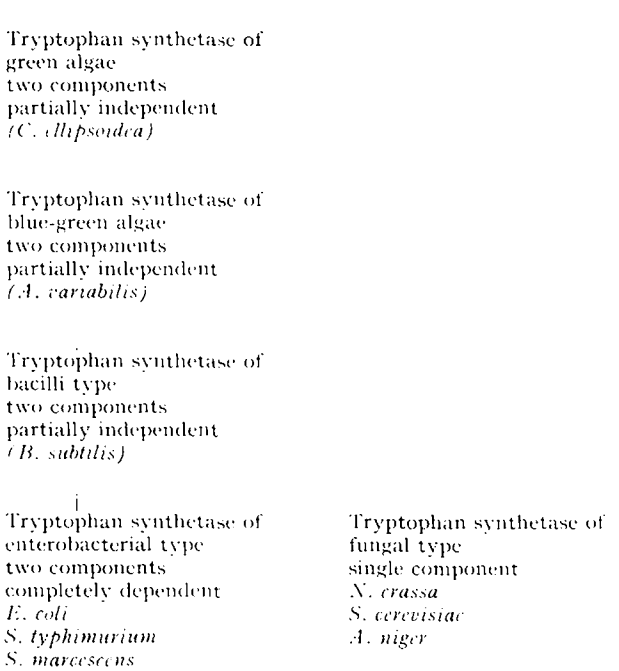

Fig. 5. Evolutional relationship of tryptophan synthetases obtained from algae, bacteria, and fungi.

verified on the lysine biosynthetic pathway by VOGEL[26], on phosphoglucomutase by JOSHI *et al.*[27], on cytochrome *c* by MARGOLIASH AND SMITH[28], on dehydrogenases by KAPLAN[29] and on aldolase by RUTTER[30]. The tryptophan synthetase also contributes support to this principle.

This map coincides on many points with the phylogenetic studies based on morphological taxonomy[31,32], but several new features arise. First, the blue-green algae are morphologically close to bacteria, and had been considered apart from green algae which is doubtless an ancestor of higher plants. However, present results show that the tryptophan synthetase from both algae can complement each other much better than bacterial tryptophan synthetase does, and the two algal proteins are highly related in many points. Similar results were obtained from the biochemical taxonomy of the lysine biosynthetic pathway by VOGEL[26].

Secondly, Bacillus or probably other gram-positive bacteria have a tryptophan synthetase more related to the algal one than to the enterobacterial enzyme. This may suggest a group of ancestral bacteria to algae.

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