THE EFFECT OF FREE AMINO ACIDS AND RELATED COMPOUNDS ON THE ACTIVITY OF PLANT ENZYMES. II.*

INHIBITION OF TRYPTOPHAN SYNTHASE (4.2.1.20) FROM THE PLANTS OF *Pisum sativum* L. by AMINO ACIDS AND GROWTH SUBSTANCES

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The inhibition of partially purified tryptophan synthase of young *Pisum sativum* plants (pea) with 13 amino acids, tryptamine, and growth regulators (3-indolylacetic acid, β-(3-indolyl)propionic acid, α-naphthylacetic acid gibberellic acid, chlorocholine chloride, α,β-dichlorobutyric acid and allyl isothiocyanate) has been investigated and the results compared with those from Part I of this series. Cysteine in higher concentration is a strong inhibitor, while at lower concentration it is an activator. Proline inhibits weakly and acts mainly repressively, threonine has mainly an inhibitory effect. Tryptophan synthase is strongly inhibited by tryptophan, appreciably by tyrosine, while other amino acids and tryptamine display only a weak effect. Growth regulators also inhibit weakly but allyl isothiocyanate more strongly. K_m of tryptophan synthase was found for DL-serine, indole, pyridoxal phosphate. The type of inhibition was determined as well as K_i for DL-threonine and L-cysteine.

The effects of intracellular substances, especially amino acids and their metabolites and hormonal substances, on the activity of plant tryptophan synthase and the kinetics of the reactions have not yet been thoroughly investigated. The activity of tryptophan synthase in living leaves of beans is strongly decreased by infiltrated threonine and proline in low concentration, and by relatively high concentrations of asparagine and γ-aminobutyric acid; on the other hand when in low concentration cysteine and asparagine increased the activity[1]. Tryptophan synthase from the seeds of *Cicer arietinum* was strongly inhibited by cysteine[2]. L-tryptophan and DL-tryptophan decreased the activity of tryptophan synthase in cell-free homogenates of *Escherichia coli*[3] and in purified preparations from the roots and above-ground parts of pea plants[4], it decreased the biosynthesis of tryptophan if added to the cultivation medium for microorganisms[5,6]. It also repressed in *Coprinus radiatus*[7], *Neurospora crassa*[8], and *Escherichia coli*[12]. The activity of tryptophan synthase was decreased by glycine and threonine in the extracts from the seeds of *Cicer arietinum*[2], by threonine in the homogenates from kale[9], and by homologs and derivatives of tryptophan in microorganisms[5,10]. Of plant hormones β-indolylacetic acid inhibited purified tryptophan synthase from *Cicer arietinum*[11] to 10%, in *Neurospora crassa* it had no inhibitory effect and did not act repressively either[8], while β-(3-indolyl)propionic acid inhibited[11] 100%; in *Escherichia coli*

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both acids strongly induced the biosynthesis of tryptophan synthase[12]. No data are available from the literature on the effect of artificial hormonal substances on the tryptophan synthase activity or on the type of its activity. Although tryptophan synthase is not an allosteric enzyme[13-16] its regulation is very important because it is a key enzyme in the biosynthesis of tryptophan and indolic plant growth hormones.

Free amino acids (especially proline, threonine, *etc.*) and growth substances are important for the regulation of enzymatic activity. In the case of tryptophan synthase scant evidence is available as to which of these substances act as inhibitors-activators and which as inductors-repressors. In Part I their effect in living plant was followed.

In this paper the results are presented of experiments where the effect of 13 amino acids, tryptamine and some natural and artificial growth regulators on the activity of partly purified tryptophan synthase of pea plantlets was investigated. Induction-repression and inhibition-activation by single substances is discussed and the kinetics of the inhibitors, threonine and cysteine, is presented.

EXPERIMENTAL

Plants. Tryptophan synthase was prepared from the above-ground parts of 14 days old plantlets of pea (*Pisum sativum* L., cv. Raman) of 1970 harvest. The plants were cultivated in daylight in a greenhouse on hotbed soil. All experiments were carried out with an enzyme partly purified on Sephadex. The preparation of the acetone powder, extraction and purification of the enzyme and incubation were carried out according to Chen and Boll[4]. All preparative operations were carried out at 0 to +2°C.

Preparation of the acetone powder: The plants were rinsed with distilled water and homogenized with a five-fold amount of cooled acetone (−20°C) for one minute, the homogenate was filtered on a precooled Büchner funnel and washed with the same amount of acetone. The acetone powder obtained was predried in a current of air under the hood for 30 minutes, then thoroughly dried in a desiccator over $CaCl_2$ and stored in darkness in a closed vessel at −20°C.

Purification of enzyme: The acetone powder was extracted with a ten-fold amount of 0·1M phosphate buffer of pH 8·0 (ref.[17]) for 30 minutes and the mixture centrifuged at 22000 *g* for 10 minutes. The supernatant was filtered through a Sephadex G-50 superfine column and the enzyme separated and eluted with a 0·02M phosphate buffer[4] of pH 8·0 (ref.[17]) at a 8 $ml/cm^2/h$ flow-rate which was regulated with a peristaltic pump. For the determination a 6—9 ml fraction of the eluate was used as enzyme preparation[4].

Determination of activity and enzyme inhibition: The activity of the enzyme was determined in test tubes on a water bath at 30°C according to Chen and Boll[4] (with slight modification). The incubation mixture of 1 ml total volume contained: 60 μmol of DL-serine, 0·2 μmol of indole, 80 μmol of potassium phosphate buffer of pH 8·0, 10 μg of pyridoxal phosphate, and 0·4 ml of the enzyme preparation. In the control variant indole was omitted, while in the blank enzyme was substituted by the corresponding buffer. The reaction was stopped after 90 minutes incubation, during which the rate (v_0) did not change, by addition of 0·1 ml of 10% KOH. The enzyme activity was determined on the basis of indole content decrease (according to Greenberg and Galston[18]), with the difference that the wave-length used for spectrophotometry was 575 nm[17]. The inhibitors were added to the mixture in 0·1 ml aqueous solution volumes.

The Michaelis constants were determined graphically according to Lineweaver and Burke[19]

and K_i were calculated for competitive inhibitors according to equation

$$K_i = i/(K_p/K_m) - 1 ,$$

where i is molar concentration of the inhibitor, K_m is Michaelis constant and K_p modified Michaelis constant in the experiment with the inhibitor; for non-competitive inhibitors the following equation was used

$$K_i = i/(V/V_p) - 1$$

in which V is maximum reaction rate and V_p is modified maximum reaction rate in the experiment with the modifier[19]. The results of the experiments are presented as arithmetical averages of three parallel experiments.

All chemicals used were of analytical grade purchased from Lachema. The centrifugation was carried out on a cooled Janetzki K 24 centrifuge, spectrophotometrical measurements on a SF 4 spectrophotometer.

RESULTS AND DISCUSSION

Michaelis constants derived graphically by the method of Lineweaver and Burke[19] are: for DL-serine $K_m = 0.65 . 10^{-2}$M, for indole $K_m = 1.26 . 10^{-4}$M, and for pyrido-

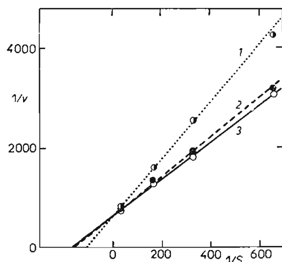

FIG. 1

Graphical Derivation of the Michaelis Constant (K_m) of Tryptophan Synthase of *Pisum sativum* for DL-Serine and the Type of Inhibition with DL-Threonine According to Lineweaver and Burke

3 without Inhibitor; *2* with 2·5 μmol of DL-threonine/ml; *1* with 25 μmol of DL-threonine/ml; *v* starting reaction rate in μmol indole per minute at experimental conditions; *S* concentration of serine in mol.

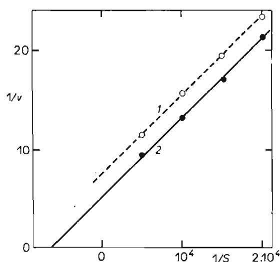

FIG. 2

Graphical Derivation of K_m of Tryptophan Synthase of *Pisum saticum* for Indole and the Deduction of the Type of Inhibition with DL-Threonine According to Lineweaver and Burke

2 without Inhibitor; *1* with 25 μmol of DL-Threonine/ml; *v* starting reaction rate in μmol of indole per minute at experimental conditions; *S* concentration of indole in mol.

xal phosphate $K_m = 0{\cdot}97 \,.\, 10^{-5}$M (Figs 1–3). The values for indole and pyridoxal phosphate are practically identical with those from the literature[11,13].

In our first communication we found that many amino acids increase or decrease the activity of tryptophan synthase in living leaves[1] but the type of the change of this activity was not determined. In this paper the activating or inhibiting effect of DL-alanine, DL-threonine, L-cysteine, glycine, L-asparagine, γ-aminobutyric acid, L-proline, L-hydroxyproline, L-methionine, L-tyrosine, DL-phenylalanine, L-leucine, L-tryptophan and tryptamine were followed. The concentration of the substances used and the degree of inhibition (activation) are shown in Table I.

L-Cysteine in 10^{-2}M concentration strongly inhibited tryptophan synthase, but already in a 10^{-3} concentration it activates it weakly (Table I). This inhibition is competitive with respect to DL-serine (Fig. 4), with $K_i = 2{\cdot}04 \,.\, 10^{-3}$M, and non-competitive with respect to indole, with $K_i = 1{\cdot}11 \,.\, 10^{-2}$M (Fig. 5). If these results are compared with those of the Ist communication it may be supposed that in the living plant the appreciable increase of tryptophan biosynthesis from indole and serine, produced by L-cysteine[1], is caused by prevailing induction. However, further proofs have to be made.

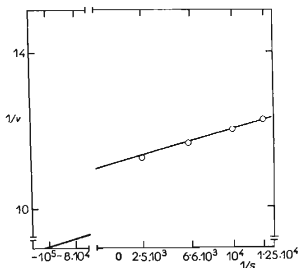

FIG. 3

Graphical Derivation of K_m of Tryptophan Synthase of *Pisum sativum* for Pyridoxal Phosphate According to Lineweaver and Burke

v Starting reaction rate in μmol of indole per minute, at experimental conditions; S concentration of pyridoxal phosphate in mol.

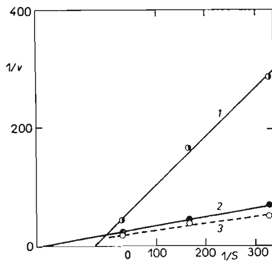

FIG. 4

Graphical Derivation of the Type of Inhibition of Tryptophan Synthase of *Pisum sativum* with L-Cysteine, to DL-Serine as Substrate, According to Lineweaver and Burke

v Starting reaction rate in μmol of indole per minute, at experimental conditions; S concentration of DL-serine in mol; *2* without inhibitor; *1* with 10^{-2} L-cysteine; *3* with 10^{-3}M L-cysteine.

L-Threonine in a $2{\cdot}5 \,.\, 10^{-1}$M concentration inhibited pea tryptophan synthase to 19% (Table I) which practically agrees with the degree of biosynthesis decrease in living bean leaves[1], in homogenates of kale leaves[9], and with the degree of inhibition of tryptophan synthase from the seeds of *Cicer arietinum*[2]. It is evident that threonine has only an inhibitory effect. This inhibition is competitive with respect to DL-serine (Fig. 1), with $K_i = 3{\cdot}9 \,.\, 10^{-3}$M and uncompetitive with respect to indole[20] (Fig. 2), which is in agreement with the kinetic properties of tryptophan synthase[14-16].

L-Proline inhibits pea tryptophan synthase weakly only at higher concentrations (Table I). In view of the strong decrease of tryptophan biosynthesis in living bean leaves[1] it is evident that it acts mainly as a repressor. A similar represor activity is also exerted by γ-aminobutyric acid which in a 10^{-2}M concentration inhibits negligibly (Table I), but in the living leaf decreases tryptophan biosynthesis strongly[1].

L-Asparagine of relatively high concentration (10^{-2}M) inhibits tryptophan synthase rather strongly. At a low concentration (10^{-4}M) it inhibits weakly (Table I), while when it is infiltered into the leaves it activates tryptophan biosynthesis[1] at low concentrations and inhibits it at a higher concentration. Tryptophan is a strong inhibitor of tryptophan synthase in plants (Table I) as has already been shown by many authors[2,4,5,8,13]. In the same concentration (10^{-3}M) tryptamine inhibits negligibly (Table I), which is in agreement with the results obtained with *Neurospora crassa*[8]. Tyrosine already inhibits pea tryptophan synthase at a low concentration (10^{-4}M), DL-alanine and L-methionine inhibit weakly, glycine, L-hydroxyproline, L-leucine, and DL-phenylalanine do not inhibit at 10^{-2}M concentration (Table I), although glycine strongly inhibited tryptophan synthase from the seeds of *Cicer arietinum*[2]. It is an enzyme of rather different properties.

3-Indolylacetic acid and β-(3-indolyl)propionic acid inhibit tryptophan synthase at a relatively high concentration weakly (Table I) which is in agreement with the

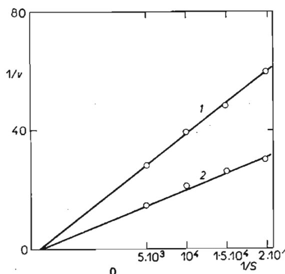

FIG. 5

Graphical Derivation of the Type of Inhibition of Tryptophan Synthase of *Pisum sativum* with L-Cysteine, to Indole as Substrate, According to Lineweaver and Burke

v Reaction rate given by the consumption of μmol of indole per minute at experimental conditions; *S* concentration of indole in mol; 2 without inhibitor; 1 with 10^{-2} ML-cysteine.

results of Delmer and Mills[13], but in the case of β-(3-indolyl)propionic acid our results differ from those of Nair and Vaidyanathan[11] who found a 100% inhibition of tryptophan synthase from *Cicer arietinum*. Gibberellic acid also inhibits tryptophan synthase weakly, while kinetin has no effect (Table I). Among bioactive substances of *Brassicaceae* allyl isothiocyanate inhibits tryptophan synthase from pea plantlets in a 10^{-3}M concentration quite strongly (Table I), while the technical growth regulators, α,β-dichlorobutyric acid and chlorocholine chloride, inhibit weakly (Table I).

TABLE I

Inhibition and Activation of Tryptophan Synthase of *Pisum sativum* by Amino Acids and Growth Regulators

Substance	Concentration, mol/l	% Inhibition (−) or activation (+)
DL-Alanine	10^{-3}	− 5·5
L-Cysteine	10^{-2}	±51·0
	10^{-3}	+ 5·5
Glycine	10^{-2}	0·0
γ-Aminobutyric acid	10^{-2}	− 1·5
L-Asparagine	10^{-2}	−11·0
	10^{-4}	− 5·2
L-Hydroxyproline	10^{-2}	0·0
L-Proline	$2{\cdot}5 . 10^{-1}$	− 7·2
L-Methionine	10^{-3}	− 2·4
L-Tyrosine	10^{-4}	− 7·1
DL-Phenylalanine	10^{-3}	− 1·0
L-Leucine	10^{-2}	0·0
DL-Threonine	$2{\cdot}5 . 10^{-1}$	−19·0
	$2{\cdot}5 . 10^{-2}$	− 4·5
L-Tryptophan	10^{-3}	−13·9
Tryptamine	10^{-3}	− 1·4
3-Indolylacetic acid	10^{-4}	− 3·8
	10^{-8}	− 3·3
β-(3-Indolyl)propionic acid	10^{-4}	− 5·3
	10^{-8}	− 5·0
α-Naphthylacetic acid	10^{-4}	− 2·8
	10^{-8}	− 2·4
Gibberellic acid	10^{-4}	− 5·0
	10^{-8}	− 4·7
Kinetin	10^{-5}	± 0·0
Chlorocholine chloride	10^{-3}	− 2·5
α,β-Dichlorobutyric acid	10^{-3}	− 1·4
Allyl isothiocyanate	10^{-3}	−12·3

From the analysis of the results of Part I and II of this series it is evident that many amino acids have an inhibitory effect on tryptophan synthase, but some have a predominantly repressive effect; many of them increase tryptophan biosynthesis at low concentrations (induction, activation) and decrease it at higher concentrations (repression, inhibition). Changes in concentrations of free amino acids in the course of ontogenesis and under the effect of the surrounding medium[21] represent a very important cause of changes in the activities of enzymes.

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