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

EFFECT OF DL-THREONINE, L-PROLINE, γ-AMINOBUTYRIC ACID, L-ASPARAGINE AND L-CYSTEINE ON THE ACTIVITY OF TRYPTOPHAN SYNTHASE (4.2.1.20) IN LIVING BEAN LEAF (*Phaseolus vulgaris* L.)

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The activity of tryptophan synthase (L-serine hydro-lyase, incorporating indole; 4.2.1.20) in living bean leaves (*Phaseolus vulgaris* L., cultivar Perlička) is very high. By increasing the concentration of some free amino acids introduced by vacuum infiltration, the activity is altered. Proline inhibits it pronouncedly, threonine less and γ-aminobutyric acid still less so. Asparagine at a low concentration (10^{-3}M) activates tryptophan synthase slightly but at higher concentrations (10^{-1}M) it is inhibitory. The enzyme is activated by cysteine, more powerfully at increased concentrations. It was shown that changes in the content of individual free amino acids in living plant leaves affect rather substantially the enzyme activity, particularly that of tryptophan synthase which is one of the enzymes involved in the indole hormone biosynthesis.

The composition and quantitative relations of free amino acids in plant organs affect rather substantially the direction and the intensity of metabolic pathways. Many amino acids display inhibitory effects on enzymes of many plants as well as of other organisms[1,2]. Some amino acids accumulate in plants under conditions unsuitable for growth, in particular proline, γ-aminobutyric acid and others[3,4]. The quantitative ratio of amino acids also differ, such as that of serine to threonine[3].Threonine inhibits the growth of potatoes[5] and tryptophan synthase of plants[5,6]; it hastens the ripening of cotton plant pods[7]. Serine is a substrate of the enzyme; tryptophan gives rise to plant indole hormones[1]. Serine stimulates pronouncedly the formation of potato tubers[5] and retards the ripening of cotton plant pods[8], apparently due to the effect of enhanced formation of indole hormones. The ratio between serine and threonine (*i.e.* the ratio of substrate and inhibitor of tryptophan synthase) is an indicator of the intensity of growth of plants and of their organs[3-5], of the length of the vegetation period of plant cultivars[3,8,9]. Early cultivars possess a higher serine/threonine ratio in their leaves which is apparently associated with the activity of tryptophan synthase and the intensity of biosynthesis of indole hormones[3]. The ratio of serines to threonine also decreases pronouncedly in plant organs during ontogeny as growth is inhibited, the lowest values being found in seeds[3,8].

Some amino acids, on the other hand, are activators of biochemical processes. *E.g.*, aspartic acid and asparagine stimulate the attachment of graft to stock[10], in breeding tubers they increase the formation of tubers[5] and the like. This is apparently due to the fact that they act as precursors of nucleic acids and also by influencing the activity of plant enzymes.

To verify Štefl's theory of intracellular inhibition and activation of enzymes by endogenous amino acids represented in varying amounts among the free amino acids experiments are under way to test the effect of individual amino acids and related compounds on the activity of plant enzymes, in particular of those of plant hormone biosynthetic pathways. In the present communication we describe the results obtained with DL-threonine, L-proline, L-cysteine, L-asparagine and γ-aminobutyric acid, introduced by vacuum infiltration, on the activity of tryptophan synthase in bean leaves.

EXPERIMENTAL

The activity of tryptophan synthase was determined by vacuum infiltration into cotyledons and young leaves of the bean (*Phaseolus vulgaris* L.), cultivar Perlička, harvest 1968, degree of breeding V_2, from the breeding station Šibřina.

The plants were grown in hotbed soil in pots 10 cm in diameter (three plants in each). For the 1st experiment they were sown on August 26, 1969, the enzyme activity being assayed on September 16, 1969 (experimental variants 1—8); for the 2nd experiment they were sown on October 14, 1969, the enzyme being assayed on October 30, 1969 (variants 9—15); for the 3rd experiment they were sown on December 8, 1969, the enzyme being assayed on January 7, 1970 (variants 16—21).

For the experiments we removed leaves as well as young healthy primary leaves since they displayed the same tryptophan synthase activity. The leaves were immediately thoroughly rinsed in tap water, dried with filter paper and sectioned into 1 cm^2 segments. After immersion of 4 g amounts in distilled water or in freshly prepared solutions (depending on the variant) cooled to 2°C, vacuum infiltration was carried out (30 min evacuation, 1 min infiltration of solutions). The zero point of reaction was read at the moment of infiltration. Three replicates were carried out in each variant. After infiltration, the samples were washed with distilled water, blotted with a filter paper and spread on moist filter paper in a Petri dish. In closed Petri dishes they were incubated in diffuse daylight at 25—27°C for 4 h. The experimental variants are shown in Table I.

After incubation, the samples were covered in a beaker with 50 ml boiling 96% ethanol. After 1 h of standing the material was homogenized with a small amount of crushed glass in a mortar, the homogenate was filtered, the precipitate was washed with 75% ethanol. The amino acids from the filtrate were trapped on Katex S (a sulfonate cation-exchange resin of Czechoslovak production) in an H^+ form and eluted from it with 4M-NH_4OH (250 ml). The eluate was evaporated and the dry residue was dissolved in 2 ml 10% isopropyl alcohol. Tryptophan was separated from the other amino acids by chromatography on Whatman No 2 paper with 0·10M HCl and estimated with ninhydrin[11]. From every sample 3 chromatographic estimations were carried out. The enzyme activity is reported in mg synthesized tryptophan per g fresh leaf weight.

Free amino acids in bean leaves were determined after incubation in the same residues as tryptophan using the ninhydrin method in two-dimensional chromatograms[11].

Nitrogen was determined by the microdiffusion technique[12] after mineralization of samples (6—8 replicates); the dry weight at 105°C (3 replicates).

The following chemicals were used: DL-serine (Reanal), L-proline (C. Erba), DL-threonine, L-cysteine, L-asparagine, indole, γ-aminobutyric acid (Lachema), pyridoxine (Central Health Service, Prague).

TABLE I

Experimental Variants and Dry Weight and Nitrogen Contents of Bean Leaves

Infiltrated solution K: 10^{-3}M indole + 10^{-2}M DL-serine + 2 . 10^{-4}M pyridoxine. Incubated for 4 h.

Variant No	Infiltrated solution	Dry weight %	% Nitrogen in dry weight ± maximum deviation
1	water		
2	K		
3	K + DL-threonine 5 . 10^{-2}M		
4	K + DL-threonine 10^{-2}M	10·16 ± 0·04	6·42 + 0·34
5	K + DL-threonine 5 . 10^{-3}M		−0·32
6	K + L-proline 5 . 10^{-2}M		
7	K + L-proline 10^{-2}M		
8	K + L-proline 5 . 10^{-3}M		
9	water		
10	indole 10^{-3}M + B_6 (2 . 10^{-4}M) + threonine 10^{-1}M		
11	K + L-cysteine 10^{-4}M		
12	K + L-cysteine 10^{-3}M		
13	K + L-asparagine 10^{-3}M	9·84 ± 0·03	7·12 + 0·15
14	K + L-asparagine 10^{-2}M		−0·14
15	K + L-asparagine 10^{-1}M		
16	water		
17	K		
18	K + cysteine 10^{-4}M		
19	K + γ-aminobutyric acid 10^{-4}M	11·09 ± 0·15	6·46 + 0·35
20	K + γ-aminobutyric acid 10^{-3}M		−0·22
21	K + γ-aminobutyric acid 10^{-2}M		

RESULTS AND DISCUSSION

Table I shows the experimental variants, the dry weight and nitrogen content in bean leaves used for the experiments. The optimal concentrations of indole and of serine were determined in preliminary experiments. The concentrations of infiltrated amino acids were chosen so that after infiltration their amount exceed to different extent the contents of other amino acids, particularly of serine, the substrate of tryptophan synthase.

Bean leaves contain relatively little free amino acids. Vacuum infiltration introduces relatively high amounts of amino acids (Table II). In this way, the reaction rate of enzyme biosynthesis of tryptophan is substantially increased, together with the

TABLE II

Content of Some Amino Acids in Bean Leaves

The values are shown in per mill of fresh leaf weight after incubation. The variants are described in Table I.

Amino acid	Variant No		
	1	2	3
Aspartic acid	25·05 ± 0·94	27·03 ± 0·75	12·18 ± 0·15
Glutamic acid	17·29 ± 0·46	13·82 ± 0·41	11·12 ± 0·14
Serine[a]	12·36 ± 1·36	36·13 ± 0·33	36·15 ± 0·25
Threonine	6·97 ± 0·77	6·73 ± 1·00	191·13 ± 0·53
Alanine	6·70 ± 0·39	7·95 ± 0·22	6·67 ± 0·01
Proline	1·40 ± 0·06	4·10 ± 0·19	6·81 ± 0·55
Valine	4·42 ± 0·64	3·59 ± 0·06	4·07 ± 0·37
γ-Aminobutyric acid	3·45 ± 0·03	7·38 ± 0·31	7·68 ± 0·31

[a] Serine was determined together with glycine which was present in traces.

inhibition of tryptophan synthase. Serine was infiltrated in such amounts that in the course of incubation at most 25% of it was used up in the leaf. In all the variants, serine was infiltrated into bean leaves in equal relative amounts while the amounts of threonine and proline were proportional to the concentration of infiltration solutions (Table II, variants 3–8). The content of the other free amino acids varied very little as compared with the variant infiltrated with distilled water (Table II).

In an illuminated living leaf the effect of concentration of the individual free amino acids on tryptophan synthase activity is very pronounced.

Activity of Tryptophan Synthase

Bean leaves infiltrated with solutions of serine, indole and vitamin B_6 contain a high activity of tryptophan synthase. After 4 h of incubation in the light it increased by 620% as compared with the variant infiltrated with water (Table III, variants 1, 2, 16, 17). In a living leaf the enzyme activity is much greater than in homogenates[5,6]. A great role is apparently played here by light[13].

Effect of Threonine on Tryptophan Synthase Activity

Infiltrated threonine inhibits relatively strongly the activity of tryptophan synthase[5,6] (Table III). According to the assumed reaction mechanism a competitive inhibition should be involved[5]. The results of this experiment are quite different, however.

TABLE II
(*continued*)

Variant No				
4	5	6	7	8
24·59 ± 0·12	25·63 ± 0·33	17·95 ± 1·32	24·04 ± 1·00	29·56 ± 0·06
11·17 ± 0·09	19·51 ± 1·34	17·36 ± 1·30	21·31 ± 0·00	20·97 ± 0·11
42·04 ± 1·13	34·67 ± 1·53	36·60 ± 1·00	35·02 ± 0·04	39·56 ± 0·88
44·41 ± 0·95	26·17 ± 1·25	4·33 ± 0·27	4·95 ± 0·52	4·57 ± 0·57
7·57 ± 0·03	8·66 ± 0·39	10·84 ± 0·32	9·72 ± 0·31	8·56 ± 0·15
3·59 ± 0·30	6·66 ± 0·43	128·67 ± 2·01	32·22 ± 0·10	10·82 ± 0·04
3·02 ± 0·01	2·36 ± 0·43	2·54 ± 0·28	3·52 ± 0·37	3·82 ± 0·72
5·47 ± 0·20	7·50 ± 0·31	10·70 ± 0·50	9·54 ± 0·91	8·57 ± 0·06

Threonine inhibited most at the lowest concentration used ($5 . 10^{-3}$M, Table III, variant 5), the effect being much less at the higher concentrations (Table III, variants 4, 3, 10). If a competitive inhibition were the mechanism, the variant with low substrate and high threonine concentration (No 10) should be particularly strongly affected. The cause of the opposite effect was not identified.

The inhibition of tryptophan synthase by threonine supports the assumption[3,5] that free threonine, even at a low concentration in the presence of other amino acids in a living plant inhibits tryptophan synthase and thus apparently blocks the biosynthesis of the plant indole hormones. In the leaves of rapidly growing young beans the ratio of serine to threonine[3,5] was relatively high (1·77; Table II, variant 1) but if threonine was infiltrated, the ratio dropped to 0·94 (variant 4, Table II), together with a pronounced decrease of tryptophan synthase activity (Table III, variant 4 and 5). This supports the view of Štefl[4,5] that the serine/threonine ratio which changes particularly with changes in growth intensity and which serves to a considerable extent as an indicator of plant growth, is related to the activity of tryptophan synthase. According to experiments[3,4] the ratio does not drop below 0·3 so that the extreme case, such as that produced in variant 10 (Table III) is unlikely in a living plant. In other experiments with homogenates of marrow-stemkale[5] we observed inhibition of tryptophan synthase of approximately the same degree as in the present experiments.

TABLE III

Activity of Tryptophan Synthase in Living Bean Leaves after Infiltration of Some Amino Acids

The enzyme activity is expressed in per mill of synthesized tryptophan per fresh leaf weight (± maximum deviation) and in per cent referred to the variant infiltrated with water (*a*) or with serine and indole (*b*). The variants are described in Table I.

Variant No	Tryptophan[a]	% Control	
		a	*b*
1	3·13 + 0·06 − 0·07	16·21	100
2	19·30 + 1·14 − 1·46	100	616·61
3	14·67 + 0·95 − 1·15	76·01	468·69
4	14·28 + 1·09 − 0·90	73·98	456·23
5	12·58 + 1·77 − 1·29	65·18	401·91
6	9·75 + 1·31 − 1·10	50·51	311·50
7	10·61 + 0·43 − 0·55	54·97	338·97
8	10·52 + 2·13 − 1·36	54·50	336·10
9	1·53 + 0·10 − 0·18	15·40	100
10	7·97 + 0·31 − 0·52	80·03	520·91
11	10·33 + 1·36 − 1·12	103·99	675·16
12	11·76 + 1·07 − 0·62	118·38	768·62
13	10·92 + 1·43 − 1·01	109·92	713·72
14	9·67 + 0·64 − 1·09	97·34	632·02
15	7·94 + 0·14 − 0·21	79·93	518·95
16	2·65 + 0·50 − 0·65	16·02	100
17	16·54 + 2·16 − 2·13	100	624·15
18	17·20 + 0·90 − 1·22	103·99	649·05

TABLE III
(*continued*)

Variant No	Tryptophan[a]	% Control	
		a	*b*
19	16·49 + 0·17 − 0·17	99·69	622·26
20	15·52 + 1·42 − 0·81	93·83	585·66
21	14·10 + 0·85 − 0·34	85·24	532·07

[a] Arithmetic mean of 3 replicates.

Effect of Proline

L-Proline supplied to bean leaves inhibited powerfully the tryptophan synthase even at a very low concentration (at 5 . 10^{-3}M by 54%; Table III, variant 8). Further increase of proline concentration enhanced the inhibition only slightly (Table III, variants 7, 6). In young bean leaves the proline concentration is very low (Table II, variant 1) and hence it will show very little inhibition. These results and relationships confirm that proline as a pronounced inhibitor of plant growth[3] is a powerful inhibitor of tryptophan synthase. Free proline accumulates in plants whenever growth is suppressed for any reason[3,14,15] which is accompanied by decrease hormone formation and decreased activity of tryptophan synthase.

Effect of Cysteine

Nair and Vaidyanathan[6] found in experiments with homogenates that cysteine is a pronounced inhibitor of tryptophan synthase. In the present experiments with plant leaf homogenates[5] we observed, however, that cysteine is on the contrary an activator of this enzyme. This was confirmed in the experiment where at a high concentration of cysteine in the infiltration solution (10^{-3}M) (Table III, variant 12), the amino acid was a clear activator of tryptophan synthase (118% of the control with water). At lower concentrations of cysteine the activation was very low (Table III, variant 11, 18). The results agree with the finding that the content of cysteine in plants during ontogeny is increased[16], particularly before flowering[3] when also the activity of tryptophan synthase is highest[17].

γ-Aminobutyric Acid and Asparagine

γ-Aminobutyric acid also acts as an antimetabolite[3] even if less pronouncedly than proline. Tryptophan synthase in living bean leaves is inhibited only minutely at low and medium concentrations (Table III, variant 19, 20) and appreciably only at very high concentration (10^{-2}M, Table III, variant 21). Such an enormous increase in the level of free γ-aminobutyric acid is common in some plants, such as in winter rape before flowering[4].

At low concentrations (10^{-3}M) asparagine stimulated very slightly, at higher ones (10^{-2}M) it had no effect and at very high ones (10^{-1}M) it inhibited sligtly the effect of tryptophan synthase (Table III, variants 13, 14, 15). Asparagine belongs to amino acids which can suppress enzyme activity only at very high levels, this being apparent in hibernating plants[4,14] when the high concentration of free amino acids contributes to the suppression of physiological processes.

The assumption of Štefl[3-5] was borne out here that the activity of tryptophan synthase as the central enzyme of the biosynthesis of plant indole hormones is in a living plant substantially inhibited by threonine and proline even at a very low concentration while cysteine and asparagine at low concentrations slightly stimulate it. The assumption is supported that the ratios and amounts of these amino acids in the living leaf may play a role in the regulation of enzyme processes and hence in the growth and development of plants.

The inhibitory effects of some amino acids on tryptophan synthase must be taken into account when assying the activity of this enzyme in living material and in crude homogenates. In those cases the activities do not correspond to the enzyme present normally but to the altered enzyme at the given concentrations of metabolites, especially of amino acids.

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