Metabolism of Aromatic Amino Acids in Plants I. On 3, 4-dihydroxyphenylalanine in *Stizolobium hassjoo*

by Atsushi KOMAMINE*

Received March 29, 1962

Various plant parts of *Stizolobium hassjoo* become remarkably brown when injured. 3, 4-dihydroxyphenylalanine contained in the plant is the agent responsible for the browning when induced by polyphenoloxidase, as proved by Hattori and M. Shiroya¹⁾. They found also that 3, 4-dihydroxyphenylalanine (dopa) crystallized out of the exudate from the cut surface of the tip of stem on drying. This fact suggests a high content of dopa in this plant and dopa seems to occupy a peculiar position in the metabolism of aromatic amino acids in *Stizolobium hassjoo*. In order to approach to the physiological significance of dopa in this plant, I made studies on the distribution of dopa in some leguminous plants and in various organs of *Stizolobium hassjoo* and *Vicia faba*, and on the change in the content of dopa and related substances during germination. Biogenesis of dopa was also studied.

Materials and Methods

Extraction. Materials were extracted with 10% acetic acid on a boiling water bath. Gaseous sulfur dioxide was introduced into the solvent during extraction to prevent the oxidation of dopa.

Paper chromatography. Whatman No. 1 filter paper was used throughout. Phenol-water (8:2 w/w) and butanol-acetic acid-water (4:1:2 v/v) were used as solvents. Ninhydrin, potassium ferricyanide and Pauly's reagent²⁾ were used for the detection of dopa.

Estimation of dopa. Dopa was estimated by a modified quantitative paper chromatographical method, the principle of which is based on the linear relationship between the logarithm of the spot area on chromatogram and that of the amount of substance³⁻⁶⁾. Narrow strips of filter paper, 12 mm in width and 400 mm in length, were used. A definite volume of the sample solution, taken in a micropipette, was put onto a square between 4 and 5 cm from one end of the paper strip. After developing in phenol-water till the solvent front ran a distance of 30 cm, the strip was dried in an oven at 80° for 40 min. One % potassium ferricyanide in butanol saturated with water (adjusted to pH 8) was sprayed on the strip, which was dried at room temperature overnight. The distribution of dopa on the paper was determined by spectrophotometer equipped with an attachment for paper chromatogram at 570 m μ . The area which gave more than 0.1 in absorbance per 1 mm in width was taken as a spot. Since spot width was constant, spot length could be regarded as spot area in this case. As shown in Fig. 1, the relationship between the logarithm of spot length and that of the amount (concentration in this case) of dopa was nearly linear.

Estimation of protein and total nitrogen. Material was boiled in 80% ethanol and crushed in a mortar, then extracted five times with ten times its volume of boiling

* Department of Botany, Faculty of Science, University of Tokyo.

80% ethanol for 1 hr. More than 97% of free amino acids were extracted by this procedure. Resulting pulp was dried in an oven at 100° for 5 hrs. and then in a desiccator overnight. Its nitrogen content, which could be regarded as protein nitrogen in this case, was determined by micro-Kjeldahl technique. The total nitrogen content in the material dried in an oven at $60 \pm 5^\circ$ for 10 hrs. and then in a desiccator for a week was also determined by the micro-Kjeldahl technique.

Cultivation of embryo. Etiolated seedlings, the epicotyls of which were 0.6 cm long in an average, were decotyledonated. The decotyledonated embryos were cultivated aseptically in test tubes with nutrient solution (four times diluted Knop's solution plus 5% glucose), added with 0.025% tyrosine or phenylalanine solution.

Infiltration of the test solution into epicotyls. Epicotyls, which were cut off from etiolated seedlings, were infiltrated in a vacuum with aqueous solutions of tyrosine, phenylalanine and alanine, separately. Infiltrated epicotyls were floated in water for 24 hrs. in the darkness.

Experiments with C^{14} -labelled tyrosine. Epicotyls, which were cut off from etiolated seedlings, were put in a solution of C^{14} -labelled tyrosine. The epicotyls were kept in the darkness at 25° for 5 days, being supplied, from time to time, with distilled water. In this way, each gram of epicotyl received about 0.187 mg of C^{14} -labelled tyrosine (53,100 cpm). In a few days, samples were killed by being dropped into boiling 70% ethanol. After complete extraction of the compounds soluble in aqueous ethanol, a portion of the concentrated extracts was applied to two dimensional paper chromatography run in butanol-acetic acid-water, followed by phenol-water. The radioactivity of another portion was measured by radiation counter.

Preparation of the crude oxidase from etiolated seedlings. Epicotyls of etiolated seedlings (146 g) were crushed in a blender with 100 ml of tris-maleic acid buffer. The brei obtained was filtered by suction. About two times its volume of acetone were added at 0°. The precipitate formed was centrifuged and dried in a desiccator. The dried preparation was used as the crude oxidase.

Fig. 1. Relationship between logarithm of concentration and that of spot area (spot length in this case) of dopa on chromatogram.

Results

Distribution of dopa.

The distribution of dopa in 21 species was examined by paper chromatography. The results are summarized in Table 1. Dopa could be detected in *Stizolobium*, *Mucuna*, *Vicia* and *Cytisus*.

Dopa contents in the various organs and tissues of *Stizolobium hassjoo* and *Vicia faba* were determined. The results are shown in Table 2. *Stizolobium hassjoo* has a higher content of dopa than *Vicia faba*. Dopa contents per dry weight in young tissues are higher than those in mature tissues in every organ of both plants.

Other free amino acids in the various organs and tissues of *S. hassjoo* were also

Table 1. Distribution of dopa in some leguminous plants.

Species	Organ*	Dopa
<i>Arachis hypogaea</i>	S, HS, RS and CS.	—
<i>Canavalia ensiformis</i>	S, HS, RS and CS.	—
<i>Cassia tora</i>	St and L.	—
<i>Crotalaria sessiliflora</i>	S.	—
<i>Cytisus scoparius</i>	St and L.	+
<i>Dolichos lablab</i>	S, HS, RS and CS.	—
<i>Erythrina americana</i>	L.	—
<i>Glycine max</i>	S, HS, RS and CS.	—
<i>Lespedeza bicolor</i> var. <i>japonica</i>	St and L.	—
<i>Phaseolus chrysanthos</i>	S, HS, RS and CS.	—
<i>Phaseolus nanus</i>	S, HS, RS and CS.	—
<i>Phaseolus vulgaris</i>	S, HS, RS and CS.	—
<i>Pisum sativum</i>	S, ES, RS and CS.	—
<i>Sophora angustifolia</i>	St and L.	—
<i>Stizolobium hassjoo</i>	S, ES, RS, CS, St, L, P, Pe, Pi and Sta.	+
<i>Mucuna deerlingianum</i> **	S, ES, RS and CS.	+
<i>Mucuna utilis</i> **	S, ES, RS and CS.	+
<i>Trifolium repens</i>	St and L.	—
<i>Vicia faba</i>	S, ES, RS, CS, St, L and P.	+
<i>Vigna sinensis</i>	S.	—
<i>Wistaria floribunda</i>	S and P.	—

* S: seed, HS; hypocotyl of seedling, RS; root of seedling, CS; cotyledon of seedling, ES; epicotyl of seedling, St; stem, L; leaf, P; pod, Pe; petal, Pi; pistil, Sta; stamen.

** These were donated by Dr. J. D. Sampayo d'Orey, Director of Jardim e Museu agricola do Ultramar Lisboa, for which the author is grateful.

examined. Aspartic acid, glutamic acid, dopa, glycine, threonine, alanine, tyrosine, valine, leucine, lysine, histidine, arginine, proline, asparagine, stizolobic and stizolobinic acid¹⁴⁾ were detected. The distribution pattern found in dopa was similar to those of asparagine and tyrosine in leaves and stems: more amount of asparagine and tyrosine was found in young tissues than in mature ones. Phenylalanine was distinctly detected only in the unripe seeds. Stizolobic and stizolobinic acid were detected only in seeds and seedlings of *Stizolobium* and *Mucuna*.

Change in the content of dopa and its related substances during germination.

To make clear the metabolic aspect of dopa in the seedlings of *S. hassjoo*, the change in the content of dopa and other free amino acids, dry weight, total nitrogen, protein nitrogen were examined during germination in the darkness.

In resting seeds, 16 amino acids above mentioned were detected as free. After radicles began to grow, the content of free amino acids in the seedlings increased. The increase of dopa and asparagine was most remarkable. Alanine, tyrosine, valine and leucine also increased. However, no qualitative change of free amino acids was observed during germination.

Changes in dopa content, dry weight, total and protein nitrogen were shown in Figs. 2, 3 and 4. As shown in Fig. 2, dopa content increased in every organ,

Table 2. Dopa contents in organs of *Stizolobium hassjoo* and *Vicia faba*.

Species	Organ	Percentage of dopa per fresh weight	Perc ntage of dopa per dry weight
<i>Stizolobium hassjoo</i>	Stem 1 (0-2 cm from tip)	1.7	4.8
	Stem 2 (2-15 cm from tip)	0.38	1.0
	Stem 3 (15-30 cm from tip)	0.28	0.9
	Leaf 1 (0-2 cm in length of major axis)	0.62	2.5
	Leaf 2 (5-7 cm ")	0.42	2.0
	Leaf 3 (10-15 cm ")	0.19	0.86
	Pod 1 (2.74g, average fresh weight of 20 pods)	1.1	6.7
	Pod 2 (6.12g, ")	1.2	6.5
	Pod 3 (21.20g, ")	0.19	1.1
	Unripe seed 1 (0.20g, fresh weight in one pod)	0.92	6.2
	Unripe seed 2 (0.65g, ")	0.82	5.2
	Unripe seed 3 (5.43g, ")	0.72	2.6
	Petal	0.98	—
	Pistil and stamen	1.0	—
<i>Vicia faba</i>	Stem 1 (0-2 cm from tip)	0.17	0.49
	Stem 2 (2-5 cm from tip)	0.06	0.16
	Leaf 1 (0-1 cm in length of major axis)	0.16	0.46
	Leaf 2 (1-3 cm ")	0.057	0.24
	Leaf 3 (3-10 cm ")	0.031	0.11
	Pod 1 (1.5-5 cm in length)	0.41	2.32
	Pod 2 (5-10 cm in length)	0.14	0.77

especially in epicotyl and root, paralled with elongation of epicotyl. On the other hand, corresponding to the increase of dopa content, the decrease of dry weight of cotyledon was observed (Fig. 3). As is seen from Fig. 4, the increase of dopa nitrogen was corresponding to the diminution in protein nitrogen during germination.

Bio genesis of dopa in the seedlings.

The positive relationship between the change of dopa and protein during germination suggests that the formation of dopa would have taken place at the expense of hydrolysate of protein in seeds. Thus, it was important to know the amino acids constitution of the seed protein, one of which might be the precursor of dopa. The hydrolysate of seed protein was subjected to paper chromatography. Aspartic acid, glutamic acid, glycine, alanine, threonine, tyrosine, proline, dopa in roots.

Fig. 2. Change in dopa content per 10 etiolated seedlings during germination. 1; total dopa $\times 1/2$, 2; dopa in cotyledons, 3; dopa in epicotyls, 4; alanine, threonine, tyrosine, proline, dopa in roots.

Fig. 3. Change in dry weight per 10 etiolated seedlings during germination. 1; total dopa content in mg $\times 1/20$, 2; dry weight of cotyledons in g $\times 2$, 3; dry weight of epicotyls in g $\times 4$, 4; dry weight of roots in g $\times 4$.

Fig. 4. Change in protein nitrogen per 10 etiolated seedlings during germination. 1; total dopa nitrogen $\times 15$, 2; total protein nitrogen, 3; protein nitrogen in cotyledons, 4; protein nitrogen in epicotyls $\times 2$, 5; protein nitrogen in roots $\times 2$.

valine, leucine, phenylalanine, lysine were detected. However, dopa could not be detected, although oxidation was prevented by constant stream of gaseous sulfur dioxide during hydrolysis with 20% HCl.

To make clear the biogenetic pathway of dopa in the seedlings, the following experiments were carried out.

1) Culture and infiltration experiments. Decotyledonated embryos, cotyledons and epicotyls were cultured in the solutions of tyrosine and phenylalanine, which were found in the hydrolysate of seed protein and had a close structural relation to dopa. The results are summarized in Table 3. Significant increase of dopa was observed in the case of cultivation or infiltration with tyrosine.

2) Change of amino acids in the seedlings placed in an evacuated room. The cultivation and infiltration experiments suggest that dopa might be formed from tyrosine. Since oxygen is necessary for formation of dopa from tyrosine, this pathway should be blocked under oxygen deficiency, and accumulation of tyrosine might be expected. Etiolated seedlings were divided into three groups. The first group was immediately crushed and the amino acids were extracted. The second group was placed in an atmosphere saturated with water vapour for 48 hrs. at

25° in the darkness. The third group was placed in a room evacuated with a suction pump and saturated with water vapour for 48 hrs. and then amino acids were extracted. The amino acids in three groups were compared with each other by paper chromatography and the dopa contents in three groups were also determined. The distinctive accumulation of tyrosine in the third group of seedlings was observed. Dopa contents are shown in Table 4. Total dopa content in third group was a little higher than that of the first group, but lower than that of the second group. These

Table 3. Results of culture and infiltration experiments.

Method	Organ	Culture medium* or infiltration solution	Percentage of dopa per fresh weight
Culture	Decotyledonated embryo	K. + Glucose	0.59
	"	K. + Glucose + Tyrosine	0.92
	"	K. + Glucose + Phenylalanine	0.61
	Cotyledon	K. + Glucose	0.61
	"	K. + Glucose + Tyrosine	0.67
	Epicotyl	K. + Glucose	0.25
	"	K. + Glucose + Tyrosine	0.43
	"	K. + Glucose + Phenylalanine	0.29
Infiltration	Epicotyl	Distilled water	0.22
	"	Tyrosine	0.28
	"	Phenylalanine	0.27

* K.: Knop's solution.

Table 4. Dopa content in various organs in *Stizolobium*.

Group Organ	Dopa content in mg per 10 seedlings		
	The first group	The second group	The third group
Cotyledon	130.7	167.1	148.1
Epicotyl	17.2	23.0	21.5
Root	15.6	21.3	8.5
Total	163.5	211.4	178.1

findings show that dopa formation was inhibited in the third group, but the inhibition was not complete under this condition.

3) Experiments with C^{14} -labelled tyrosine. To confirm dopa formation from tyrosine, D, L- C^{14} -2-labelled tyrosine was supplied to the epicotyls of seedlings. After incubation for 5 days, the materials were extracted with 70% ethanol. Seventy % of the given radioactivity was found in the ethanol extract. The radio-autogram of the ethanol extract is shown in Fig. 5. Since D-tyrosine was not metabolized, about half of total radioactivity of the ethanol extract was found in tyrosine spot. The radioactivity (15.8% of the activity of the ethanol extract) was found in dopa, while 33% of the radioactivity was found in an undetermined substance which gave spot 3 in Fig. 5. A small amount of radioactivity was also found in the other undetermined substances which separated as spots 4, 5 and 6 in Fig. 5.

4) Existence of the enzyme which converts tyrosine to dopa. It was intended to demonstrate the existence of the enzyme which oxidizes tyrosine to dopa. Crude enzyme was prepared from seedlings. A reaction mixture, 1 ml of 0.025% tyrosine solution plus 0.5 ml of enzyme solution, was incubated at pH 6.8 and 30°. After 30 min., the reaction mixture became red, and then brown. After 10 hrs., precipita-

Fig. 5. Autoradiogram of an extract of epicotyls supplied with C¹⁴.labelled tyrosine. The solvent of the first direction; butanol-acetic acid-water, the second one; phenol-water. 1; tyrosine, 2; dopa, 3, 4, 5 and 6; undetermined substances.

Fig. 6. O₂ uptake by the crude enzyme prepared from etiolated seedlings. 1; dopa 2; tyrosine.

tion of a melanoid substance or substances was observed. The same change in the colour of the reaction mixture was observed when dopa was incubated, although the colour became red more rapidly. No tyrosine was detected paper chromatographically in the reaction mixture after 10 hrs., when tyrosine was incubated. However, dopa could not be detected during the incubation. Probably, dopa had been oxidized to dopa quinone spontaneously and then to a melanoid substance or substances, if formed. O₂ uptake was measured with Warburg's manometer when tyrosine and dopa were incubated. The results are shown in Fig. 6.

Discussion

Dopa was at first isolated from the legumes of *Vicia faba* by T. Torquati⁷⁾ and identified by Guggenheim⁸⁾. Miller reported its occurrence in the seeds of *Mucuna pruriens*⁹⁾ and T. Yoshida found it in *Mucuna capitata*¹⁰⁾. Recently, dopa was also found in *Sarothamnus scoparius*¹¹⁾ and *Euphorbia lathyris*¹²⁾.

Table 5. Dopa content in the leaves of *Stizolobium*.

	Length of major axis of leaf in cm	Percentage of dopa per dry weight	Dopa content per 100 leaves in mg.
Leaf 1	0-2	2.5	16.5
Leaf 2	5-7	2.0	59.1
Leaf 3	10-12	0.86	198.0

Distribution of dopa was examined by paper chromatography, but it was not detected in other genera than *Vicia*, *Stizolobium*, *Mucuna* and *Cytisus*. It is likely, therefore, that dopa shows a comparatively narrow distribution in Leguminosae.

As shown in Table 2, a considerably high content of dopa was found in the various organs of *Stizolobium* and *Vicia*. Young tissue has higher content of dopa per dry weight than mature tissue in every organ. However, as is seen from Table 5, in which total dopa content per one hundred leaves is shown, the content of dopa per one mature leaf is higher than that per one young leaf. These findings suggest that dopa is produced rapidly in young tissues and accumulates in the tissues. This is consistent with the change in dopa content of etiolated seedlings during germination; it increases in parallel with elongation of epicotyls as shown in Fig. 2. Formation of dopa seems to occur in parallel with growth.

It is important to know the pathway of dopa formation for making clear the physiological significance of dopa. The formation of dopa from tyrosine was proposed in animal tissues¹³⁾, but it has not been definitely studied in plants. The results of culture and infiltration experiments suggest that dopa might be formed from tyrosine, because the significant increase of dopa was observed when tyrosine was fed. The fact that dopa formation was inhibited and tyrosine accumulated in the seedlings placed in an evacuated room, supports the existence of the pathway from tyrosine to dopa; the fact shows that the oxidative pathway from tyrosine to dopa would be blocked by oxygen deficiency. The enzyme which converts tyrosine to dopa might exist, although dopa could not be detected in the process of oxidation of tyrosine with the enzyme prepared from etiolated seedlings. Because the crude enzyme oxidizes both tyrosine and dopa, and dopa is much more rapidly oxidized than tyrosine. Therefore, it is probable that tyrosine might be oxidized to dopa and the latter should be in its turn oxidized to dopa quinone and then to a melanoid substance or substances.

Furthermore, the formation of dopa from tyrosine was confirmed by the experiments with C¹⁴-labelled tyrosine: dopa was labelled with C¹⁴ when C¹⁴-labelled tyrosine was supplied to the epicotyls of seedlings. However, it is an interesting fact that more radioactivity was found in the spot 3 representing an undetermined substance in Fig. 6, when active tyrosine was fed to epicotyls. It suggests another pathway of metabolizing tyrosine may eventually exist. The identification of the substance is being carried out.

Summary

1) Dopa was found in *Stizolobium*, *Mucuna*, *Vicia* and *Cytisus*. Distribution of dopa in leguminous plants examined is limited in them.

2) Dopa contents in various organs of *Stizolobium hassjoo* and *Vicia faba* and change in dopa content during germination in *S. hassjoo* were investigated. It could be concluded that dopa might be produced rapidly in young tissues and accumulate in the tissues.

3) Biogenetic pathway of dopa from tyrosine was confirmed by the culture and infiltration experiments, the experiments with radioactive tyrosine and others.

The author wishes to express his cordial gratitude to Prof. S. Hattori under whose guidance this study has been carried out. The gratitude is also due to Prof. M. Shimokoriyama for his valuable suggestion and encouragement.

References

- 1) Hattori, S., and Shiroya, M., *Physiologia Plantarum* **7**: 68 (1955). 2) Ames, B. N., *J. Am. Chem. Soc.* **74**: 252 (1952).
- 3) Fisher, R. B., Persons, D. S., and Morrison, G. A., *Nature* **161**: 764 (1948).
- 4) Fisher, R. B., Morrison, G. A., and Persons, D. S., *ibid.* **164**: 183 (1949).
- 5) Satake, K., and Miyaki, K., *J. Pharm. Soc. Jap.* **71**: 249 (1951).
- 6) Satake, K., and Miyaki, K., *Rep. Inst. Food Microbiol.* **5**: 64 (1952).
- 7) Torquati, T., *Arch. Farm. Sperim.*, **15**: 213 (1913).
- 8) Guggenheim, M., *Z. Physiol. Chem.* **88**: 276 (1913).
- 9) Miller, E. R., *J. Biol. Chem.* **44**: 481 (1920).
- 10) Yoshida, T., *Tohoku J. Exp. Med.* **48**: 27 (1945).
- 12) Liss, I., *Naturwiss.* **48**: 304 (1961).
- 12) Blaschko, H., *J. Physiol. (London)* **96**: 50 (1939).
- 13) Hattori, S., and Komamine, A., *Nature* **183**: 1116 (1959).

摘 要

駒嶺 穆: 植物体内での芳香族アミノ酸代謝 I. ハッショウマメにおける
3, 4-ジオキシフェニルアラニンについて

ハッショウマメ (*Stizolobium hassjoo*) に多量にふくまれ、その黒化現象の原因物質となっている 3, 4-ジオキシフェニルアラニン (ドーパ) の生理的意義を追求するため、マメ科植物におけるドーパの分布、ハッショウマメ、ソラマメのいろいろの組織におけるドーパの含有量の比較、ハッショウマメの発芽にともなうドーパおよびその関連物質の変化、ドーパの生合成経路などをしらべたところ、次のことがわかった。

- 1) ドーパは *Stizolobium*, *Mucuna*, *Vicia*, *Cytisus* 属に見いだされ、そのマメ科植物間での分布はあまり広いものではない。
- 2) ドーパの乾燥量あたりの含有量は、若い組織ほど大きく、ひとつの器官の全含有量をくらべると、古いものの方が大きい。まためばえでは上胚軸の生長と平行的にドーパはふえる。これらの事実から、ドーパは若い組織で急速につくられ、組織に堆積すると考えられる。
- 3) ドーパの生合成経路については、培養および infiltration の実験、放射能をもつチロシンをもちいた実験、酸素欠乏状態においためばえにおけるチロシン、ドーパの変化などからチロシンからドーパに至る経路が確認された。(東京大学理学部植物学教室)