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BIOSYNTHESIS OF SEROTONIN AND β-CARBOLINE ALKALOIDS IN HAIRY ROOT CULTURES OF *PEGANUM HARMALA**

JOCHEN BERLIN,†‡§ CHRISTIANE RÜGENHAGEN,†§ NORBERT GREIDZIAK,§ INNA N. KUZOVKINA,¶ LUDGER WITTE‖ and VICTOR WRAY‡

‡GBF – Gesellschaft für Biotechnologische Forschung m.b.H., Mascheroder Weg 1, D-3300 Braunschweig, Germany; §BBA – Biologische Bundesanstalt für Land- und Forstwirtschaft, D-3300 Braunschweig, Germany; ‖Institut für Pharm. Biologie, Techn. Universität, D-3300 Braunschweig, Germany; ¶K. A. Timirjazev Institute for Plant Physiology, Moscow, Russia

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Key Word Index—*Peganum harmala*; Zygophyllaceae; root cultures; suspension cultures; tryptophan decarboxylase; serotonin; β-carboline alkaloids; bioconversion.

Abstract—Tryptophan decarboxylase (TDC) activity, and serotonin and harmane alkaloids levels of a *Peganum harmala* root culture were followed over a growth cycle. A close relationship between the peak of TDC activity and the highest specific content of serotonin was found, while such a correlation was not observed for the β-carboline alkaloids, the main constituents of the root cultures. The content of serotonin, but not of the alkaloids was greatly enhanced by feeding tryptamine. Trials to identify individual biosynthetic steps of alkaloid biosynthesis by feeding were only partially successful, because only the oxidation of dihydro-β-carbolines to aromatic harmane alkaloids was observed when the various alkaloids were added to the root cultures. Tracer experiments with tryptophan revealed the presence of 5- and 6-hydroxytetrahydronorharmane in the root cultures which, however, should not be biosynthetic intermediates of the major alkaloids, harmalol and harmine.

INTRODUCTION

The biosynthetic pathway of β-carboline alkaloids in *Peganum harmala* is still based on feeding experiments to intact plantlets and has not been clarified at the enzyme level [1, 2]. Attempts to obtain suitable callus and cell suspension cultures of *P. harmala* accumulating higher amounts of β-carboline alkaloids for such studies have so far failed [3, 4]. Detectable alkaloid contents were only found in freshly initiated, slowly growing and aggregated cultures [5]. When alkaloid, and in parallel, serotonin formation were enhanced in such cultures by media variation, high increases of tryptophan decarboxylase (TDC) activity were noted [4]. In rapidly growing cell suspension cultures however, TDC activity was no longer present or inducible and, consequently alkaloid and serotonin formation ceased [2, 6]. By selection for 4-methyltryptophan tolerance, variants with TDC activity and serotonin levels were recovered from cell suspensions lacking TDC and serotonin [6]. However, the biosynthesis of β-carboline alkaloids was not restored by the selection of TDC-rich cells, indicating that other enzymes are rate-limiting in this pathway. With the establishment of stable, good-growing hairy roots accumulating high levels of β-carboline alkaloids and serotonin [7, 8], a useful system now seems to be available for studying the interrelationship of the two pathways.

A general advantage of cell suspension culture systems is that they are often easier to manipulate. In particular enzymes linking primary and secondary metabolism, such as phenylalanine ammonia lyase [9, 10], tryptophan decarboxylase [11, 12] or tyrosine decarboxylase [13], were often found to be induced after transfer of the cells to fresh medium, to a production medium or by elicitation. Whether such alterations of activities indicate that an enzyme is indeed a regulatory control of the derived pathway is not yet clear. In the case of suspension cultures of *P. harmala* a close correlation between TDC activity and derived metabolites was shown for serotonin, but not for the β-carboline alkaloids [6]. Thus, it was of interest to see whether a closer relationship between TDC activity and alkaloid levels would be detected in the root cultures where the alkaloids are the major constituents [8].

RESULTS AND DISCUSSION

The accumulation of serotonin and β-carboline alkaloids and the pattern of tryptophan decarboxylase activity of the hairy root culture PH A4 over a growth cycle are shown in Fig. 1. The specific alkaloid contents were usually unchanged over the growth cycle. From many independent measurements over a 3-year period, it became evident that variations had to be accounted for by differences in the root material rather than from growth

*Part 3 in the series 'Hairy Root Cultures of *Peganum harmala*'. For Part 2 see ref. [8].

†Author to whom correspondence should be addressed.

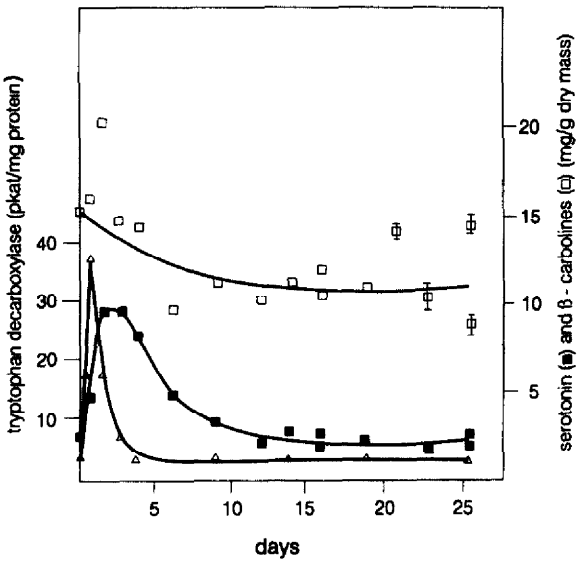

Fig. 1. Pattern of tryptophan decarboxylase activity, and of serotonin and β-carboline alkaloid accumulation, in hairy root culture PH A4 during a culture period of 4 weeks. Initial inoculum was 2 g fr. wt in 70 ml medium; final biomass was 15–18 g fr. wt, corresponding to 1.2–1.5 g dry wt.

Table 1. Relationship between inducibility and activity of tryptophan decarboxylase and accumulation of metabolites derived therefrom in cell suspension (PHF and variant line PH2) and a hairy root culture PH A4 of *P. harmala*

Line	PHF	PH2	PHA4
Biomass increase (-fold)	5.2	4.1	2.3
Initial specific content (% dry mass)			
Serotonin	0.12	1.02	0.82
β-Carbolines	0.01	0.03	1.90
TDC (pkat mg^{-1} protein)			
0 hr	0.3	2.8	2.1
24 hr	56.2	144.2	38.1
144 hr	0.3	38.1	5.8
Increase of products (μg per flask)			
Serotonin	321	932	608
β-Carbolines	18	19	672

The initial inoculum from 14-day-old cultures was 0.5 g fr. wt in 20 ml, corresponding to 25–35 mg dry wt per flask.

cycle-specific responses. In contrast, the specific yield of serotonin always showed a maximum 2–6 days after transfer to fresh medium. Thus, the accumulation pattern of serotonin indicated a close relationship with the induction of TDC activity during the first two days in the fresh medium. An induction peak of TDC activity was always noted during the first two days. However, the height of this induction peak depended upon the preculture and decreased with increasing inoculum sizes. The highest specific TDC activities in this highly productive root culture never exceeded 30–40 pkat mg^{-1} protein. TDC induction in the roots seems to be rather low if one compares it with inductions observed in suspension cultures. In Table 1 a low-producing, but still inducible suspension culture (PHF), a highly-productive 4-methyltryptophan-tolerant variant line PH2 (selected 5 years ago [6]) and the hairy root culture PH A4 are compared with respect to TDC induction and product accumulation. Evidently the degree of TDC induction is not necessarily a reliable indicator for the extent of formation of tryptamine-derived metabolites (Table 1). More important for the production characteristics of the lines seems to be the maintenance of a certain level of TDC activity over the growth cycle. Indeed, in the highly productive variant PH2, as well as in the root culture, a much higher TDC activity was maintained than that in the low-producing line (Table 1). Despite the fact that tryptamine is regarded as a precursor for both serotonin and β-carboline alkaloids [1], only serotonin formation seemed to be stimulated by the transient enhancement of TDC activity. While a rather close correlation between TDC activity and serotonin levels was evident for all three lines, this was not seen for TDC activity and β-carboline alkaloid production. This was already known for suspension cultures, but was not expected for the root cultures producing more β-carbolines than serotonin. If there is any relationship between TDC activity and β-carboline content, this may only be detected when the TDC activities of true variant lines with different β-carboline alkaloid contents are available.

For some suspension cultures it is known that nutrients are sometimes rapidly absorbed from the medium (more than necessary for growth) and that the stored nutrient is then distributed to the dividing cell population [14]. Since osmotic stress is sometimes known to induce enzymes of secondary metabolism and secondary product formation, one could imagine that the different uptake of nutrients may cause different osmotic situations in suspension and root cells. We tested this idea by studying the uptake of phosphate and carbohydrate. When grown on B5 or MS medium (1.2 mM phosphate) PH A4 had absorbed 40%, while the same inoculum of suspension cells of PH2 and PHF had taken up 40–50% within 48 hr. The sugar levels in the media of the root and suspension culture differed after 4–6 days when the biomass production of the suspension line became distinctly higher than that in the root culture. Thus, one has to conclude that the stronger enzyme induction seen in suspension cells is not likely to be due to a different rate of uptake of nutrients, and hence is not caused by different osmotic (stress) conditions.

An important question was whether the root culture would be a good system for obtaining further insights into the biosynthetic sequence of β-carboline alkaloid formation. It is still not known at what stage hydroxylation at position 7 of β-carbolines occurs. We first tested whether feeding of tryptophan and tryptamine might help increase alkaloid and serotonin production (Table 2). As the alkaloids are the main constituents of the root cultures

Table 2. Effect of feeding tryptophan and tryptamine for 2 weeks on growth, and serotonin and β-carboline alkaloids levels of hairy root cultures of *P. harmala*

Added compound	Dry wt (mg)	Serotonin (mg per flask)	β-Carboline alkaloids (mg per flask)
Control	130	0.5	1.8
2 mg tryptophan	140	0.6	1.8
5 mg tryptophan	110	0.3	1.4
10 mg tryptophan	54	0.3	0.6
10 mg tryptamine	130	6.3	1.2
20 mg tryptamine	160	10.1	1.4

Initial inoculum 0.5 g in 35 ml B50-medium.

Table 3. Feeding of β-carboline alkaloids to root cultures for 36 hr

Added compound	Harmalol	Harmaline	Harmol	Harmine
	Fold increases over control			
Harmalol	1.2	0	2.4	0
Harmaline	0	32	0	2.5
Harmol	0	0	15	0
Harmine	0	0	0	6

Initial inoculum 1 g in 10 ml medium. Amount of alkaloid hydrochlorides added 5 mg per flask. Final alkaloid contents per flask in the untreated control cultures were harmalol 240 μg, harmaline 15 μg, harmol 85 μg and harmine 560 μg.

under most culture conditions one might assume that tryptamine and tryptophan would be more efficiently incorporated into the alkaloids than into serotonin. This was not the case. When tryptamine was fed to the root cultures there were large increases in serotonin while alkaloid levels were unaffected. These effects were independent of the age of the culture and the time period of feeding. Thus, the root culture strongly resembles the suspension culture where feeding of tryptamine to even none or low producing lines led to high increases of serotonin [15, 16]. Specific serotonin contents of 5–6% can easily be obtained by feeding tryptamine to root cultures without affecting growth. As noted for suspension cultures of *P. harmala*, high tryptamine-5-hydroxylase activity is also present in root cultures and seems to mobilize nearly all fed tryptamine for immediate hydroxylation. The amounts of tryptamine in tryptamine-fed cells were very low (2–5% of the absorbed compound). The anticipation that tryptophan might be channelled to a greater extent into the β-carboline biosynthetic pathway was not observed. Tryptophan was fed at various concentrations and on various days of the growth cycle for periods between 1 and 14 days. However, under no culture conditions were any significant increases of alkaloids noted. Even serotonin levels were not distinctly enhanced by feeding tryptophan. Thus, despite the fact that the root cultures contain higher levels of TDC than low-producing suspension cultures, TDC activity must be regarded as rate-limiting, at least for serotonin biosynthesis. Feeding of higher amounts of tryptophan was also rather toxic to the cells. When alkaloids were fed to the root culture (Table 3) a distinct increase of harmine and harmol was only observed after feeding harmaline and harmalol, respectively. These reactions were not seen when the alkaloids were fed to suspension cultures of *P. harmala* producing no or low levels of alkaloids (data not shown). However, Nettleship and Slaytor showed the incorporation of radio labelled harmaline into harmine in alkaloid-producing callus cultures [3]. Thus, aromatization of dihydro-β-carboline alkaloids is the final step in this sequence. The conversion of harmalol to harmol was, however, rather low, as evidently large portions of harmalol disappeared by incorporation into the residue. Indeed, after extraction of root cells the residue of the root cultures had the same yellow fluorescence as harmalol, indicating that a large portion of the harmalol remains bound to cell walls. Feeding experiment did not give any indication of the methylation of harmol or harmalol to harmine or harmaline, respectively. Thus, the question at what stage *O*-methylation occurs needs to be checked by *in vitro* assays.

As feeding of higher amounts of tryptamine and tryptophan did not enhance alkaloid levels significantly and thus provided no clues about the biosynthetic sequence, more sensitive radio tracer experiments with tryptophan and tryptamine were performed (Table 4). After 36 hr, 85% of the radioactivity had been absorbed and 25% of the absorbed radioactivity was initially extractable with methanol. Only 16% of the absorbed radioactivity could be extracted with methanol after the 120 hr feeding period. The decrease of extractable radioactivity was mainly due to the disappearance of unchanged tryptophan and the turnover of serotonin, both being moved into the unextractable fraction. The decrease of the initial

Table 4. Per cent distribution of radioactivity in β-carbolines (harmine, harmaline, harmol, harmalol, ruine and 5-hydroxytetrahydronorharmane) and serotonin after feeding of tryptophan-3-[^{14}C] for 36, 72 and 120 hr to a 7-day-old root cultures of *P. harmala*

Compound	Per cent distribution of radioactivity after 36 hr	72 hr	120 hr
Harmine	6.2	15.2	21.9
Harmaline	0.2	0.4	0.4
Harmol	0.3	0.6	0.7
Harmalol	4.3	8.7	4.6
5-Hydroxytetrahydronorharmane	7.8	3.3	5.9
Serotonin	80.7	70.3	65.9
Ruine	0.5	1.5	0.6

The sum of the label found in these compounds was set to 100% accounting for 30–50% of the total radioactivity in the methanolic extract.

specific levels of serotonin seen during every growth period (Fig. 1) results from dilution by growth and by its turnover. Normally, harmine was the most highly labelled β-carboline alkaloid and labelling was almost completed during the first 36 hr. The apparent further increase of the radioactivity in harmine (Table 4) was only a relative increase due to the decrease of radioactive serotonin in the extract. Consequently the ratio of β-carboline alkaloids to serotonin changed gradually from 1.9:1 to 2.3:1. Considering that the β-carboline alkaloids are the main indolic constituents of *P. harmala* root cultures, the labelling of the harmane alkaloids by tryptophan-3-[^{14}C] was rather low. At best, 1–1.5% of the added tracer was found in the major alkaloid harmine, accounting for up to 60% of the total β-carboline alkaloids. When tryptamine was added as tracer to the root cultures (data not shown), the alkaloids showed even less label, as nearly all exogeneously added tryptamine was immediately hydroxylated to serotonin.

In addition to serotonin and the known β-carboline alkaloids further apparently unrelated bands were labelled when tryptophan and tryptamine were fed to the root cultures. Of particular interest was a band located on TLC plates between serotonin and harmalol, as sometimes this was the second most highly labelled zone in short period experiments (24–48 hr). A second more weakly labelled band, running very close to harmalol, was also isolated. Both bands were identified as hydroxytetrahydronorharmane by NMR and by GC-MS. From comparisons with the NMR spectra of serotonin and harmalol it is deduced that the major and more highly labelled band (lower R_f on TLC) is 5-hydroxytetrahydronorharmane, while the upper band is 6-hydroxytetrahydronorharmane. As these components have not been described previously as constituents of *P. harmala* or other β-carboline-containing plants [17], it was initially thought that they may be artefacts. It is well known that feeding of tryptophan or tryptamine may lead to the formation of norharmane and harmane, even in cultured plant cells which do not form harmane alkaloids or in autoclaved medium containing tryptophan or tryptamine [18, 19]. However, the tetrahydronorharmanes with their specific hydroxylation pattern were also isolated as minor components from unfed root cultures. Presently there is no indication that the tetrahydronorharmanes were formed during extraction. Thus, despite the assumption of Allen and Holmstedt [17] that some of the reported naturally occurring β-carbolines found in plants may indeed represent artefacts, the norharmane derivatives must be regarded as minor constituents of *Peganum* hairy root cultures. It is unlikely that the hydroxytetrahydronorharmane alkaloids are precursors of the harmane alkaloids.

As only hydroxylated norharmane derivatives were isolated the question at what stage the hydroxyl group is introduced at the 7-position of the β-carboline alkaloids is again of interest. Though Nettleship and Slaytor [3] failed to demonstrate incorporation of 6-hydroxytryptophan and 6-hydroxytryptamine into harmane alkaloids of callus cultures with low productivity and Stolle and Gröger [20] postulated 1,2,3,4-tetrahydroharmane-1-carboxylate as first intermediate, we searched carefully for the 6-hydroxyl derivates of tryptophan and tryptamine in the highly productive root cultures. From these experiments, however, it must be assumed that the stationary concentration of 6-hydroxytryptamine is either extremely low in the high-producing root cultures or the compound as such is not an intermediate in the alkaloid biosynthesis. A search by GC-MS for 6-hydroxytryptophan of a partially purified amino acid fraction (with labelled tryptophan as internal standard) of the root cultures was also negative. As feeding experiments with the highly productive root cultures evidently do not provide more clues for the verification of the proposed pathway of β-carboline alkaloid biosynthesis than the low producing callus and suspension cultures, future research must be directed toward the proposed enzyme reactions [1].

EXPERIMENTAL

Cell cultures. Initiation and maintenance of the hairy root line PH A4 [7, 8], as well as selection and maintenance of the 4-methyltryptophan-tolerant cell line PH 2 [6], have been described. PHF is a 5-year-old lumpy suspension culture, derived from an untransformed root culture of *P. harmala* [21], maintained on MS medium with 2 μM 2,4-D. Growth and feeding expts were performed in vols from 10 to 70 ml per flask as indicated in the text.

Measurement of tryptophan decarboxylase (TDC) activity. TDC was isolated from 2–4 g fr. wt and purified by $(NH_4)_2SO_4$ pptn and filtration on a Sephadex PD-10-column as described in ref. [22]. In the case of low activities, the extract was concd in a Centricon-30 microconcentrator. Enzyme activity was measured by extracting labelled tryptamine into EtOAc as described in ref. [22]. Protein was determined by the Bradford method [23].

Measurement of nutrients and metabolites. Pi in the medium was measured by the phosphomolybdate method [24], sucrose and glucose enzymatically by the Boehringer test kit. Serotonin and β-carboline alkaloids were determined by the fluorescence assay of ref. [25] or by HPLC-analysis as described in ref. [8]. Distribution of radioactivity from tracer expts was measured on silica gel TLC plates ($CHCl_3$–MeOH–NH_4OH, 35:15:1; 40:10:1; 45:5:0.25; 48:2:0.25) by autoradiography and by scintillation counting of zones removed from the plates.

Isolation and identification of hydroxytetrahydronorharmanes. Freeze-dried roots (50 g) were extracted ×3 with MeOH. The extracts concd to 30 ml were chromatographed on a silica gel column (50 × 2.5 cm) by stepwise elution with $CHCl_3$–MeOH (4:1, 3:1, 1:1 and 2:3). The frs between serotonin and harmalol were combined and co-chromatographed (silica gel TLC, $CHCl_3$–MeOH–NH_4OH, 4:1:0.1) with the unknown labelled compounds derived from feeding of tryptophan or tryptamine. The zones corresponding to the label were eluted with MeOH and separately purified further on

small RP18-columns (Baker) by stepwise elution with H_2O–MeOH (100:0, 3:1, 1:1, 0:100). The identity of the compounds was deduced from ^{1}H NMR and GC-MS data. The chemical shifts and relative order in the ^{1}H NMR spectra of the aromatic three-proton spin systems allowed the determination of the position of the hydroxyl substituent by reference to the spectra of serotonin and harmalol. The presence of a $CH_2 \cdot CH_2 \cdot X \cdot CH_2$-group in both compounds was evident from 2D ^{1}H COSY spectra. The difference in chemical shifts and signal form between the two arose presumably through differences in soln conditions. The M_rs were confirmed by GC-MS analysis after silylation with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide. ^{1}H 1D and 2D (COSY) NMR spectra were recorded on Bruker AM 300 and AM 600 NMR spectrometers locked to the major D resonance of the solvent CD_3OD. The GC-MS system consisted of a chromatograph fitted with a 30 m × 0.32 mm fused capillary column coated with the methylsilicone stationary phase DB-1 (J&W Scientific). He was used as carrier gas. Conditions: inj. 250°, split 1:20; temp. prog. from 150–300°, 6° min^{-1}. The capillary column was coupled directly to a quadrupole Finnigan MAT 4515 mass spectrometer. EI-spectra were recorded at 40 eV in combination with the Incos data system.

5-Hydroxytetrahydronorharmane. ^{1}H NMR (protonated form, CD_3OD, 300 MHz). δ7.16 (*d*, H-7, J(7–6) = 8.7 Hz), 6.85 (*d*, H-4, J(4–6) = 2.3 Hz), 6.70 (*dd*, H-6), 4.24 (*br s*, H-10), 3.41 (*br t*, H-12, J(12–13) = 5.7 Hz), 2.91 (*br t*, H-13). GC-MS (di-TMSi derivative): RI = 2365 (*m/z*%) 332 (53), 317 (10), 303 (95), 288 (25), 243 (2), 73 (100).

6-Hydroxytetrahydronorharmane. ^{1}H NMR (CD_3OD, 600 MHz) δ7.21 (*d*, H-4, J(4–5) = 8.2 Hz), 6.54 (*dd*, H-5, J(5–7) = 2.3 Hz), 6.46 (*d*, H-7), 3.77 (*m*, H-12A), 3.68 (*m*, H-12B), 3.61 (*d*, H-10A, J(10A–10B) = 12.4 Hz), 3.47 (*d*, H-10B), 2.42 (*ddd*, H-13A, J(13A–13B) = 13.0 Hz, J(13A–12A, 12B) = 4.7, 7.1 Hz), 2.34 (*ddd*, H-13B, J(13B–12A, 12B) = 8.4, 8.4 Hz). GC-MS (di-TMSi derivative) RI = 2312 (*m/z*/%) 332 (50), 317 (10), 304 (58), 288 (30), 243 (23), 73 (100).

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