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BIOSYNTHESIS AND METABOLISM OF GRAMINE IN *LUPINUS HARTWEGII*

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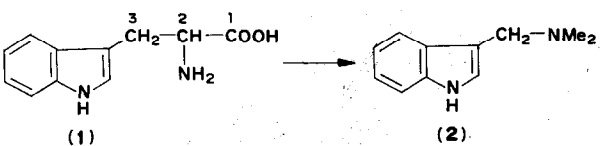
Key Word Index—*Lupinus hartwegii*; Leguminosae; lupin; gramine; tryptophan; indole-3-aldehyde; alkaloid biosynthesis; alkaloid metabolism.

Abstract—The administration of L-tryptophan-[3-^{14}C] to *Lupinus hartwegii* (3-day-old seedlings and 8-week-old plants) resulted in the formation of gramine-[*methylene*-^{14}C], indicating that gramine is produced by the same biosynthetic route in this species as in barley. Radioactive indole-3-aldehyde, labelled specifically on its aldehyde carbon, was isolated from the 8-week-old plants. However no significant amount of this compound was detected in 7-day-old seedlings, and it is suggested that indole-3-aldehyde is formed by the metabolism of gramine in the maturing plant.

INTRODUCTION

Gramine (**2**) was first isolated from the leaves of germinating barley (*Hordeum vulgare*) [1] a member of the Gramineae. This simple indole alkaloid has also been found in other members of this family: the giant reed, *Arundo donax* [2], and reed canary grass *Phalaris arundinacea* [3, 4]. The maples (Aceraceae): *Acer saccharinum* [5] and *A. rubrum* [6] also contain gramine. More recently it has been found along with the sparteine-type alkaloids in the lupins (Leguminosae): *Lupinus luteus* [7], *L. hispanicus* [8] and *L. hartwegii* [9].



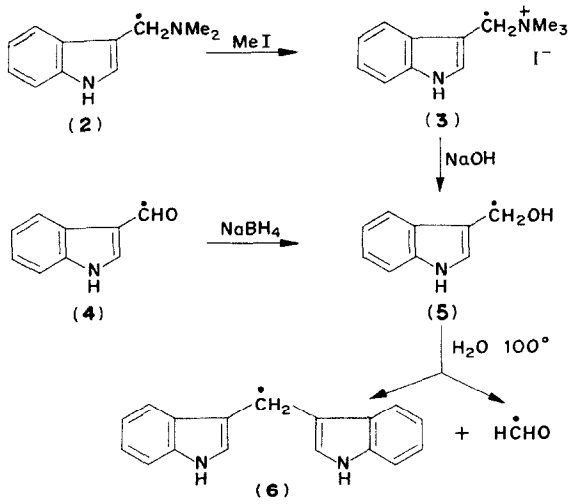
Scheme 1. Biosynthesis of gramine.

All the investigations on the biosynthesis of gramine have been carried out in germinating barley. It was of considerable interest to determine whether that tryptophan (**1**) is a precursor of the alkaloid. Later it was established that gramine formation takes place by cleavage of the tryptophan side chain between C-2 and C-3 with complete retention of all the hydrogens attached to C-3 [11, 12]. It was of considerable interest to determine whether gramine is produced in other species by the same biosynthetic mechanism as that which operates in barley. Comparatively little work has been carried out on the biosynthesis of a particular alkaloid which is occasionally found in unrelated species [13].

RESULTS AND DISCUSSION

Accordingly, L-tryptophan-[3-^{14}C] was fed by the wick method to 8-week-old *Lupinus hartwegii* plants growing in soil. The plants were harvested after 5 days and the alkaloids isolated and separated as previously described [9, 14]. A radioactive assay of a thin layer chromatogram of the crude alkaloids indicated that activity was present at a zone corresponding to gramine. On extraction this zone yielded radioactive gramine which was diluted and degraded as illustrated in Scheme 2. Gramine methiodide (**3**) was treated with NaOH in the presence of ether yielding 3-hydroxy-methylindole (**5**) [15]. 3,3′-Diindolylmethane (**6**) and formaldehyde (derived from the methylene group of gramine) were obtained on refluxing the 3-hydroxymethylindole in water. The results of this degradation are recorded in Table 2, and it is apparent that the gramine was specifically labelled on its methylene group, indicating that tryptophan is indeed a direct precursor of this alkaloid in *L. hartwegii*. There was another zone of high radioactivity on the TLC of the crude alkaloids. This zone had the

* Contribution No. 132 from this Laboratory.



Scheme 2. Degradation of gramine and indole-3-aldehyde.

same R_f as indole-3-aldehyde (**4**) and extraction yielded material identical with this substance. Dilution and degradation (Scheme 2) established that all the activity of the indole-3-aldehyde was located on its aldehyde carbon.

Indole-3-aldehyde has been detected in barley and tomato (*Lycopersicon esculentum*) shoots [16], corn (*Zea mays*) [17], cabbages (*Brassica oleracea*) [18], and a member of the Rutaceae: *Murraya exotica* [19]. It has been suggested that indole-3-aldehyde arises by an oxidation of indole-3-acetic acid or indole-3-acetonitrile [16], both these compounds being derived from tryptophan. However the levels of indole-3-acetic acid which are normally found in plants (0·0005–0·1 μg/g fr. wt) [16] are much lower than the amount of indole-3-aldehyde (4·7 μg/g fr. wt) which we found in the 8-week-old *L. hartwegii* plants. We suggest that indole-3-aldehyde is a metabolite of gramine in *L. hartwegii* plants.

Table 2. Degradation products of gramine and indole-3-aldehyde (activities in dpm/mM calculated for non-diluted alkaloids)

	Exp. 1	Exp. 2
Gramine	$1·07 \times 10^7$	$2·40 \times 10^6$
Gramine methiodide	$1·04 \times 10^7$	$2·10 \times 10^6$
3-Hydroxymethylindole	$1·08 \times 10^7$	$2·20 \times 10^6$
Formaldehyde-dimedone	$1·09 \times 10^7$	$2·40 \times 10^6$
3,3′-Diindolylmethane	$1·10 \times 10^7$	$2·35 \times 10^6$
Indole-3-aldehyde	$6·0 \times 10^7$	
3-Hydroxymethylindole	$5·85 \times 10^7$	
Formaldehyde-dimedone	$6·20 \times 10^7$	
3,3′-Diindolylmethane	$5·70 \times 10^7$	

Evidence supporting this hypothesis was obtained by feeding L-tryptophan-[3-^{14}C] to 3-day-old seedlings of *L. hartwegii*. Extraction of these seedlings 4 days later yielded radioactive gramine which was specifically labelled on its methylene group (see Table 2). However no indole-3-aldehyde was detected in these young seedlings, and no significant radioactivity was located on a TLC of the crude alkaloids at the position where indole-3-aldehyde was expected to occur. The amount of gramine (71 μg/g fr. wt) in the young seedlings was much larger than the amount found in the 8-week-old plants (5·5 μg/g fr. wt). This result clearly indicates that the gramine is being metabolized as the

Table 1. Activities of the tryptophan-[3-^{14}C] fed to *Lupinus hartwegii* and the isolated gramine and indole-3-aldehyde

	Exp. 1	Exp. 2
L-Tryptophan-[3-^{14}C]		
wt (mg)	1·20	2·04
total act. (dpm)	$2·97 \times 10^8$	$1·24 \times 10^8$
Age of plants	8 weeks	3 days
Duration of feeding	5 days	4 days
Fresh wt of plants (g)	137	127
Activity not absorbed by plants	$1·1 \times 10^5$	$1·7 \times 10^7$
MeOH extract of plants (dpm)	$9·7 \times 10^7$	$2·9 \times 10^7$
Crude alkaloids (dpm)	$2·5 \times 10^6$	$2·6 \times 10^6$
Gramine		
wt (mg)	0·76	9·0
spec. act. (dpm/mM)	$1·07 \times 10^7$	$2·40 \times 10^6$
incorporation (%)	0·016	0·12
Indole-3-aldehyde		
wt (mg)	0·64	Not detected
spec. act. (dpm/mM)	$6·0 \times 10^7$	
incorporation (%)	0·09	

plant develops. A similar change in gramine content takes place in barley as it matures [20]. The metabolism of radioactive gramine in *L. hartwegii* is being studied.

EXPERIMENTAL

General methods. A Nuclear Chicago Mark II Liquid Scintillation Counter was used for assay of the radioactive compounds using dioxane-EtOH with the usual scintillators [21].

L-*Tryptophan*-[3-^{14}C]. A small amount of the commercial material (New England Nuclear Co. Boston, Mass.) was subjected to TLC on Silica Gel PF-254 (Merck AG) developing with Me_2CO–conc NH_3 (100:1) along with gramine and tryptamine as standards. More than 99·8% of the activity was located at a spot coincident with tryptophan (R_f 0·1). However significant activity was found at the spots coincident with tryptamine (0·13%, R_f 0·65) and gramine (0·045%, R_f 0·4). Accordingly the tryptophan-[3-^{14}C] which was used in the feeding experiments was dissolved in dil. NH_3 and extracted with Et_2O for 24 hr in a continuous liquid–liquid extractor. The aq. soln was then lyophilized and the residue assayed by TLC. Negligible activity (<0·001%) was detected in the regions of the chromatogram where gramine or tryptamine would occur.

Feeding of L-*Tryptophan*-[3-^{14}C] *to* L. hartwegii *and isolation of the alkaloids.* Details of the amount and activity of the tryptophan [3-^{14}C] fed are recorded in Table 1. Seeds of *L. hartwegii* (Giant-flowered mixture) were purchased from Herbst Bros. Seedsmen, Brewster, N.Y. In exp. 1. the plants were growing in soil in a greenhouse, and the tracer was administered by means of cotton wicks inserted into the stems of the plants (8) near to ground level. In exp. 2 the seeds were germinated in glass trays containing vermiculite. After 3 days the small seedlings were removed, individually washed free of vermiculite with H_2O and placed in 30 ml beakers containing tap H_2O. About 500 seedlings were fed in this experiment. These seedlings were exposed to a bank of fluorescent lights for 16 hr/day. At the time of harvesting the plants were, on average, about 10 cm in length. The plants were macerated with MeOH (500 ml) containing conc HCl (6 ml) in a Waring blendor. After 1 day the mixture was filtered and the filtrate evaporated to small bulk (80 ml). This brown soln was made basic with NaOH and extracted with $CHCl_3$ (4 × 100 ml). Evaporation of the dried ($MgSO_4$) extract yielded the crude alkaloids. This extract was subjected to TLC on several preparative plates of Silica Gel PF-254, developing with $CHCl_3$–MeOH–conc. NH_3 (93:7:1). In this system gramine and indole-3-aldehyde have R_fs of 0·2 and 0·6 respectively. These substances were detected as dark zones when the plates were observed under short wave (254 nm) UV light. Since gramine and tryptamine (a potential metabolite of tryptophan) have almost the same R_fs in this solvent mixture, the plates were developed in a second solvent mixture: Me_2CO–conc NH_3 (100:1) when the tryptamine moves faster than gramine. However no tryptamine was detected in the extracts from *L. hartwegii*. In exp. 1 there was a dark zone coincident with indole-3-aldehyde, but none in the crude extract from the second experiment. The zones were extracted in a Soxhlet with MeOH, the amount of gramine or indole-3-aldehyde in the extracts being estimated by UV spectroscopy. Indole-3-aldehyde has a characteristic UV absorption max (in 95% EtOH) at 243, 260 and 296 nm. Gramine (in 95% EtOH) has maxima at 273, 280 and 289 nm. The extract containing gramine was diluted with inactive material, evaporated, sublimed (110°, 10^{-3} mm) and crystallized to constant activity from 50% aq. Me_2CO. The indole-3-aldehyde extract was also diluted with inactive material, evaporated, sublimed (170°, 10^{-3} mm), and crystallized from a mixture of benzene and hexane.

Degradation of gramine and indole-3-aldehyde. Gramine (244 mg) was added to MeI (10 ml) and shaken for 12 hr at 0°. The solid which separated was crystallised from MeOH affording gramine methiodide (422 mg). This methiodide (400 mg) was dissolved in H_2O (50 ml) and added to a mixture of 10% NaOH (50 ml) and Et_2O (50 ml) rapidly stirred at room temp. in a Waring blendor. After 15 min the Et_2O layer was separated and dried over Na_2SO_4. The residue obtained on evaporation was crystallised from C_6H_6–hexane affording colorless plates of 3-hydroxymethylindole (75 mg). This hydroxymethylindole (70 mg) was refluxed in H_2O (20 ml) for 10 hr, cooled, filtered and the filtrate added to a solution of dimedone (100 mg) in H_2O. On the standing overnight formaldehyde-dimedone (53 mg) separated and was crystallised from MeOH. The residue from the above filtration was dried and crystallized from C_6H_6 affording 3,3'-diindolymethane (35 mg). The radioactive indole-3-aldehyde (200 mg) was dissolved in EtOH (20 ml) and refluxed for 1 min with $NaBH_4$ (200 mg). After cooling for 1 hr, the soln was evaporated to dryness, the residue suspended in 1% NaOH, and extracted with Et_2O. The dried (Na_2SO_4) extract was evaporated and the residue crystallized from benzene affording 3-hydroxymethylindole (153 mg) which was degraded further as described under the degradation of gramine. The activities of the degradation products are recorded in Table 2.

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