ALKALOID BIOSYNTHESIS IN A *DUBOISIA* HYBRID

O. LUANRATANA[1] and W. J. GRIFFIN*

Department of Pharmacy, University of Queensland, Queensland, 4067, Australia

ABSTRACT.—The biosynthesis and metabolism of tropane alkaloids of a hybrid of *Duboisia leichhardtii* F. Muell. and *D. myoporoides* R. Br. was investigated by infiltration of 1-[U-^{14}C] ornithine, [G-^{3}H] atropine and [G-^{3}H] hyoscine into intact plants via a wick method. Ornithine was incorporated into the tropane moiety of hyoscyamine and hyoscine. The rate of incorporation was low (hyoscyamine 0.17%; hyoscine 0.13%) but of the same order of magnitude as that recorded in *Datura* spp. The specific activity of root and leaf hyoscyamine was approximately equal. The specific activity of hyoscine, which was only present in the leaves, was one half that of hyoscyamine. The infiltration of atropine over a period of 48 hours indicated a gradual increase in the activity of hyoscyamine isolated from the leaves with a smaller but similar rate of increase in hyoscine activity. This suggests metabolism of hyoscyamine to hyoscine. The infiltration of hyoscine over a period of 32 days demonstrated peak activity in the leaf hyoscine after 1 day with little variation during the test period. Final recovery of activity was approximately 10% in leaf hyoscine. Thus hyoscine appeared to undergo rapid metabolism during transportation to the leaves to give unidentified water soluble bases. The leaves act as a depot for alkaloids and their metabolites. There was no indication of translocation of activity to the roots or that hyoscine could undergo reverse metabolism to hyoscyamine.

The genus *Duboisia* incorporates three species native to Australia. *Duboisia leichhardtii* F. Muell, and *D. myoporoides* R. Br. contain tropane alkaloids and were major sources of the anticholinergic alkaloids hyoscine (also known as scopolamine) and hyoscyamine (1). In more recent times, a hybrid of *D. leichhardtii* x *D. myoporoides* has been cultivated as a preferred source.

In a previous communication (2), the variation of alkaloid in a commercial plantation of the *Duboisia* hybrid was investigated. Young hybrid plants grown from cuttings were initially observed to contain hyoscyamine as the major alkaloid; however, after about one year, hyoscine was the dominant component. From that time, especially as plants were harvested and the regrowth material analyzed, a variation in hyoscine was evident with a maximum in the late summer and spring months and a minimum in autumn when harvesting of leaf was ill-advised. The reduced hyoscine content was always accompanied by a rise in hyoscyamine. The biosynthesis and interrelationship of hyoscyamine and hyoscine has been extensively studied in *Datura* spp. where ornithine is a precursor of the tropane moiety (3) and hyoscine is derived from hyoscyamine via an intermediate 6-hydroxyhyoscyamine (4). A similar relationship between hyoscine and hyoscyamine had been demonstrated in *in vitro Duboisia* hybrid tissue culture (5). To verify this relationship and to observe any short-term variation in alkaloid, 1-[U-^{14}C] ornithine, [G-^{3}H] atropine and [G-^{3}H] hyoscine were infiltrated into stems of *Duboisia* hybrid plants using a wick method (6).

MATERIALS AND METHODS

PLANT MATERIAL.—Cuttings of the *Duboisia* hybrid were taken from a single tree[2] and after treatment with a hormone rooting powder[3] were propagated in a mist propagator. After roots had formed, the cuttings were transferred to a pot mixture containing equal parts of peat moss and gravel and nourished with 200 ml of a quarter strength Hoaglands solution (7) weekly. Otherwise each plant received 200 ml of distilled water each day. Plants were selected which developed a single stemmed structure. Prior to experimentation the plants were transferred from the glasshouse to a well ventilated room at 25°. The plants were allowed to acclimatize for 4 days receiving artificial light for 12 hours each day.

---

[1]Present address, Faculty of Science, Mahidol University, Rama VI Road, Bangkok, Thailand.

[2]A voucher specimen has been lodged with Queensland Herbarium (BRI 237629).

[3]Pyco Products, St. Mary, New South Wales.

RADIOACTIVE MATERIAL.—L-[U-^{14}C] ornithine hydrochloride[4] was available as a sterile aqueous solution with a total activity of 10μCi (285mCi/mmol).

(G-^{3}H) atropine hydrochloride was obtained as an alcoholic solution (620 mCi/mmol) at a concentration of 2mCi/ml. A volume of 5 ml was mixed with 20 mg of inactive atropine sulfate and taken up in 50 ml of 0.5N suifuric acid. The solution was made alkaline with ammonia solution (d.0.88), and the alkaloid was extracted in chloroform (3 x 5 ml). The chloroform extract was evaporated, and the residue after being dissolved in 1 ml of alcohol was neutralized with 0.1N hydrochloric acid. The volume was adjusted to 20 ml with sterile distilled water.

(G-^{3}H) hyoscine hydrobromide was prepared by catalytic exchange in a solvent with tritiated water at high specific activity. All labile tritium was removed by the manufacturer. The product was supplied as an alcoholic solution (25ml. activity 3 μCi). An aliquot of this solution (6 ml) was diluted with 30 mg of inactive hyoscine hydrobromide dissolved in 34 ml of 0.5N sulfuric acid. The solution, made alkaline with ammonia solution (d.0.88), was extracted with chloroform (3 x 5ml). The chloroform extract was evaporated, and the residue was dissolved in 2 ml of alcohol and subjected to preparative thin layer chromatography on silica gel[5] with chloroform: methanol (85:15) as the mobile phase. The hyoscine fraction was recovered after extraction of the corresponding silica gel band with alcohol. The extract was evaporated and, after resolution in 1 ml of alcohol, neutralized with 0.1N hydrochloric acid. The volume was adjusted to 10 ml with sterile distilled water.

PLANT TREATMENT.—The stem was bored 2 cm above the level of the potting mixture and a sterile cotton wick was drawn through with one end inside the stem and the other immersed in a feeding vial. After all the solution had been taken up, further volumes of 5 x 1 ml of sterile distilled water was likewise administered.

(1) *L-[U-^{14}C] ornithine hydrochloride*—The solution was diluted to 10 ml with sterile distilled water to which was added 40 mg of inactive ornithine hydrochloride. This solution was infiltrated into a single mature plant (12 months), and after 14 days leaves (fresh weight 270 g) and roots (250g) were harvested. Activity remaining in the vial was less than 1% of that a ministered.

(2) *[G-^{3}H] atropine*—A volume of 200 μl of the prepared solution was diluted to 10 ml with sterile distilled water from which 0.5 ml (13.74μCi) was infiltrated into each of 5 plants (6 months). Leaves from a single plant were collected after 8, 16, 24, 32 and 40 hours.

(3) *[G-^{3}H] hyoscine*—A volume of 200 μl of the prepared solution was diluted to 12 ml with sterile distilled water from which 1 ml (3.15μCi) was infiltrated into each of 12 plants (6 months). Groups of 2 plants were harvested as leaves and soft woody stems after 1, 2, 4, 8, 16 and 32 days. The last harvest also included roots.

EXTRACTION AND ISOLATION OF ALKALOID.—Fresh tissue was extracted with alcohol (400 ml) containing 0.4% Tween 20 in a Waring blender. The mixture was diluted with 1 liter of alcohol, mixed well and allowed to macerate for 12 hours. The mixture was filtered through cloth and the marc was pressed free of solvent and dried at room temperature. The extract was filtered and evaporated under reduced pressure to 100 ml. Excess 1N sulfuric acid was added to the concentrated extract; insoluble material thus formed was filtered off and retained. Fats and pigments were extracted from the acidified extract with 1 liter of a mixture of chloroform and ether (4:1). The defatted extract was rendered alkaline with ammonia solution, and the total alkaloids were extracted with chloroform (4 x 100 ml). The combined chloroform extracts were evaporated to give the total base residue. Water soluble bases remained in the aqueous fraction. The total base fraction was submitted to column chromatography on kieselguhr (40 g) containing 20 ml of 0.5M phosphate buffer pH 6.8. The column was developed with ether to give hyoscine; hyoscyamine was subsequently eluted with chloroform. The eluates were evaporated to yield the respective alkaloid base which was converted to the alkaloid picrate by dissolving in a minimum volume of alcohol, neutralization with 0.1N hydrochloric acid and addition of a saturated aqueous solution of sodium picrate. Alkaloid picrates were recrystallized to constant melting point and specific activity.

HYDROLYSIS OF HYOSCYAMINE.—Active hyoscyamine picrate (40 mg, sp. act. 5.3 x 10^{4}dpm/mmol) was dissolved in dilute ammonia solution and the free base extracted with chloroform; 500 mg of inactive hyoscyamine was added to the chloroform extract and the solution evaporated. The residue was refluxed with 10 ml of 10% sodium hydroxide solution for 30 min. Tropine was extracted from the reaction mixture with chloroform and afforded tropine picrate mp 270° (decomp.); sp. act. 5.1 x 10^{4}dpm/mmol (calculated allowing for diluent). The aqueous fraction was acidified, extracted with ether to give inactive tropic acid mp 116–7°.

HYDROLYSIS OF HYOSCINE.—Hyoscine was likewise hydrolyzed. Active hyoscine picrate (35 mg, sp. act. 2.0 x 10^{4} dpm/mmol) and inactive hyoscine hydrobromide (250 mg) were combined and the base was extracted with ammonia and chloroform. After evaporation, the residue was refluxed with 10 ml of 10% sodium hydroxide solution for 30 min. Oscine was extracted from the reaction mixture with chloroform and afforded oscine picrate mp 237–238°; sp. act. 2.0 x 10^{4} dpm/mmol (calculated allowing for diluent). The aqueous fraction was acidified, extracted with ether which gave inactive tropic acid mp 116–119°.

RADIOACTIVE MEASUREMENT.—Samples were counted in duplicate in a commercial scintillation mixture PCS[3]. The yellow color of picrates was decolorized by the addition of 2N hydrochloric acid (8). Other residues were digested with 1 ml of alkaline solubilizer NCS[3] for 2

[4]Amersham Australia Pty. Ltd., Ashgrove, 4060.
[5]Kieselgel, Merck.

days and decolorized by the addition of 1 ml of 30% hydrogen peroxide. The vials were stored in a cool place for 8 hrs. and 15 ml of PCS was added after 2 days. The radioactivity was measured on a Packard Tricarb® Liquid Scintillation Spectrometer (model 2425). A minimum of 10,000 counts was observed for each sample.

RESULTS AND DISCUSSION

L-[U-^{14}C] ornithine was administered to a *Duboisia* hybrid plant by a wick feeding method. After 14 days the plant was harvested and the alkaloids from roots and leaves harvested. Active hyoscyamine was isolated from both leaves (205 mg) and roots (20 mg) and had specific activities of 5.3 x 10^4 and 4.9 x 10^4 dpm/mmol respectively. Hyoscine was only isolated from the leaves and had a specific activity of 2.0 x 10^4 dpm.mmol. Hyoscyamine and hyoscine from the leaves were hydrolyzed and the activities were confined to the alkamine moieties. The incorporation of ornithine into hyoscyamine and hyoscine was low, 0.17% and 0.13% respectively, of the same order of magnitude to that experienced in the genus *Datura* (6). Thus ornithine is a precursor of the tropane moiety of hyoscyamine and hyoscine. The low incorporation of ornithine into the alkaloids could be due to general metabolism of ornithine, indicated by the high incorporation into the residual marc (8%).

(G-^{3}H)- atropine was infiltrated into a group of 5 *Duboisia* hybrid plants; leaves from a single plant were collected after 8, 16, 24, 32 and 48 hours. Hyoscine and hyoscyamine were isolated from every sample collection and the specific activities determined. The activity of hyoscyamine increased with time after infiltration, and a final incorporation of 10% of activity was achieved. The activity of hyoscine increased at a comparable rate, however, less incorporation was evident (table 1, figure 1). Excluding one collection (16 hours) the ratios

TABLE 1. Specific activity of alkaloids isolated from *Duboisia* hybrid plants infiltrated with [G-^{3}H] atropine.

Harvest time (hours)	Hyoscyamine			Hyoscine		
	Sp. act. (dpm/mmol)	Yield (g)	I%[a]	Sp. act. (dpm/mmol)	Yield (g)	I%
8	1.3 x 10^6	0.154	2.26	4.5 x 10^3	0.297	0.02
16	1.9 x 10^6	0.101	2.26	2.4 x 10^4	0.197	0.05
24	2.7 x 10^6	0.090	2.77	9.0 x 10^3	0.160	0.02
32	7.2 x 10^6	0.040	3.30	2.0 x 10^4	0.243	0.05
40	9.7 x 10^6	0.090	9.95	3.3 x 10^4	0.229	0.08

[a]Percentage incorporation = $\frac{\text{Total activity in alkaloid}}{\text{activity fed}}$ x 100.

of specific activities of hyoscyamine and hyoscine in each plant occurs at similar rates and implies a major relationship even though the recovery of infiltrated activity was low. Chemical degradation of the alkaloids was not considered since the original tritiation was general. Thus only speculative comment may be made concerning the possibility of hyoscine being a direct metabolite of hyoscyamine. However, consideration of the metabolism of exogenous hyoscyamine to hyoscine by *Duboisia* hybrid tissue culture (5) suggests it may well be so. The small quantity of d-hyoscyamine infiltrated as atropine was insufficient to affect the physical properties of those alkaloids isolated. It is assumed that metabolism occurred similar to the laevorotatory isomer.

The metabolism of hyoscine in the *Duboisia* hybrid was observed by the infiltration of [G-^{3}H] hyoscine to a total of 12 plants. Groups of 2 plants were harvested after time intervals of 1, 2, 4, 8, 16 and 32 days. The stems and leaves were collected separately. Since the low recoveries of activity in the previous

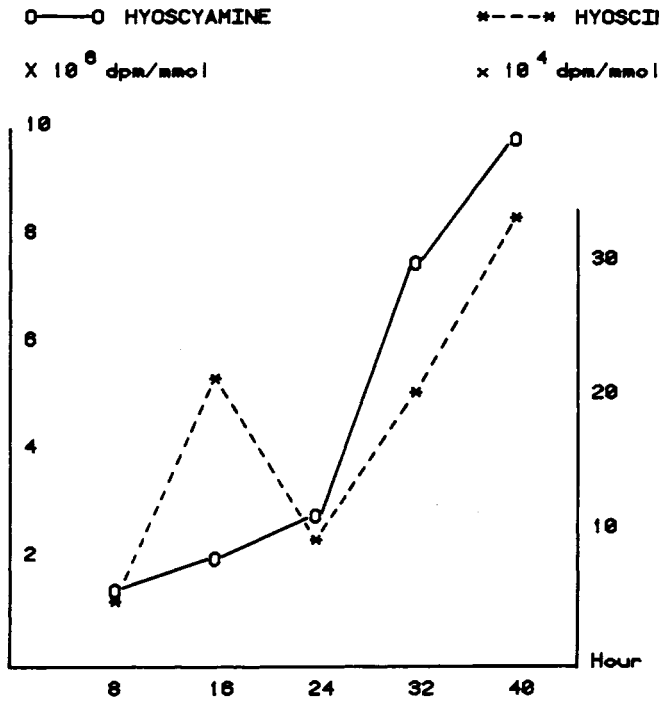

FIG. 1.

study of the metabolism of [G-^{3}H] atropine may have been due to translocation of activity to the roots, the last harvest also included roots. An extract of the total bases was made and the activity determined. The activity in the water soluble basic fractions was also measured (table 2, figure 2). The activity within

TABLE 2. Activity of alkaloid and basic fractions isolated from *Duboisia* hybrid plants infiltrated with [G-^{3}H] hyoscine.

Harvest time (day)	Sample	Total base			Hyoscine			Water sol. base	
		Activity (μCi)	Yield (g)	I%[a]	Sp. act. (dpm/mmol)	Yield (g)	I%	Activity (μCi)	I%
1	Leaf	1.23	1.800	16.8	2.5×10^6	.120	6.3	0.72	9.9
	Stem	0.25	1.380	3.4	—	—		0.00	
2	Leaf	1.68	1.843	23.0	2.3×10^6	.115	5.3	0.45	6.2
	Stem	0.12	0.757	1.6	—	—		0.00	
4	Leaf	1.64	1.727	22.5	2.3×10^6	.110	5.2	0.64	8.8
	Stem	0.05	0.493	0.7	—	—		0.00	
8	Leaf	1.77	1.974	24.2	2.4×10^6	.115	5.6	0.54	7.4
	Stem	0.03	0.106	0.4	—	—		0.00	
16	Leaf	1.91	1.653	26.2	2.2×10^6	.232	10.4	1.09	14.9
	Stem	0.01	0.129	0.1	—	—		0.00	
32	Leaf	1.50	1.612	20.5	1.4×10^6	.164	4.4	0.33	4.5
	Stem	0.01	0.773	0.1	—	—		0.00	
	Root	0.01	0.930	0.1	—	—		0.00	

[a]Percentage incorporation = $\frac{\text{Total activity of fraction}}{\text{activity fed}} \times 100.$

the stems decreased after the first day, presumably due to rapid translocation of the infiltrated hyoscine. However, hyoscine could not be detected in these extracts. Inactive hyoscine was added to the stem extract of the first harvest and, on recovery, was found to be active showing some stem activity could be

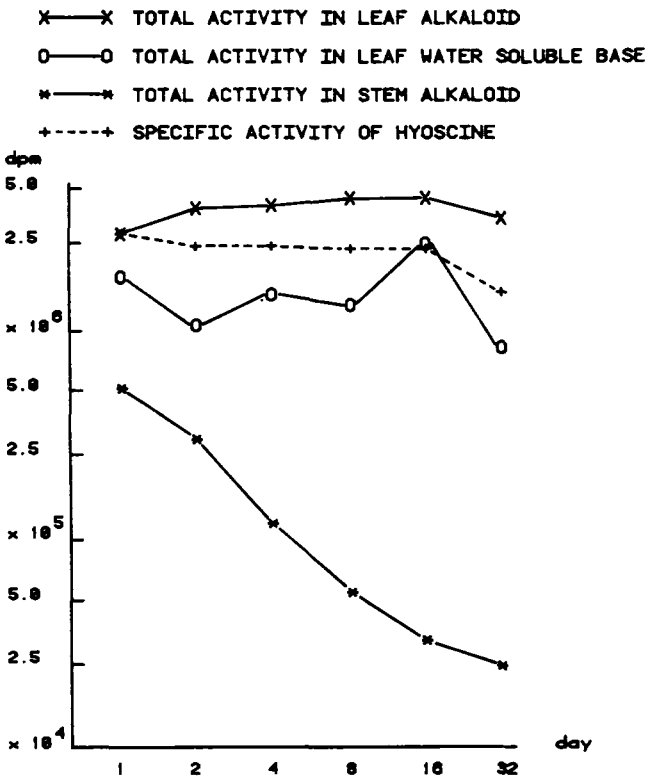

FIG. 2.

due to traces of the infiltrated alkaloid. The activity of the total basic fraction from the roots of the final collection was very low corresponding to incorporation of 0.1% suggesting the metabolism of hyoscine occurred entirely in the aerial parts. Complementary evidence was given by the observation that only those water soluble basic fractions of the leaves had substantial activity. The activity of the total bases of the leaves remained constant throughout the test period. The leaf samples were previously assayed by G.L.C. (9) and the major alkaloids, hyoscine and hyoscyamine, were isolated from an aliquot of the total basic fraction. All samples of hyoscyamine were found to be inactive showing that the metabolism of hyoscyamine to hyoscine was not a reversible reaction. This is supported by a previous publication (5) in which exogenous hyoscine was not metabolised by *Duboisia* hybrid tissue culture. However, in *Datura innoxia* ($N\text{-}^{14}CH_3$, hyoscyamine was obtained after infiltration of ($N\text{-}^{14}CH_3$) hyoscine (10). This may have resulted from transmethylation rather than specific interconversion of alkaloids. The specific activity of hyoscine was at a maximum after the first day (table 2) indicating rapid transportation from the site of infiltration. The highest recovery of the tritiated hyoscine was approximately 10%. The small variation in activity of leaf hyoscine suggests that the metabolism of hyoscine occurs during transportation within the stems and the leaves act as a depot for the unmetabolized hyoscine and its metabolites, presumably water soluble bases.

For the first time, the role of the stems in the metabolism of alkaloids within *Duboisia* has been explicitly demonstrated which explains some of the observations in recent cultivation studies (2). Experiments on the variation of alkaloid content in field cultivated *Duboisia* trees showed that the variation before the first harvest was related more to the age of the plant rather than season. The latter appeared to be more important after the first crop. In winter, hyoscine and the total alkaloid content decreased, suggesting a depression of alkaloid synthesis due to

an unfavorable environment. Also it can be conceived that during the cooler months, transpiration is reduced and the alkaloids being translocated from the roots spend more time in the stems and, hence, hyoscine is degraded to a greater degree than in the summer months when transpiration is rapid. Experiments with cytokinins on glasshouse-grown plants and, in particular, Maxicrop®[6] field trials tend to support the hypothesis that hyoscine is metabolized within the stems. The cytokinin content in the seaweed extract Maxicrop® delays leaf senescence (11, 12) thus the trees bear more leaves than untreated plants (2). This, in turn, could increase transpiration thus reducing transportation time of the alkaloid within the stems, thereby reducing hyoscine metabolism. It was shown that the reduction in hyoscine content during the cooler months would be delayed or even eliminated if sprayed with Maxicrop® (13). Further evidence for the important role of the stem in *Duboisia* cultivation was that, after the first harvest, the hyoscine content was directly related to season or month and not, in the short-term, to the age of the plant. The regrowth material contains a more uniform stem content and thus metabolism of hyoscine during translocation would be more uniform from tree to tree.

Received 30 October 1981

LITERATURE CITED

1. C. Barnard, *Econ. Bot.*, **6**, 3 (1952).
2. O. Luanratana and W. J. Griffin, *J. Nat. Products*, **43**, 552 (1980).
3. F. A. Turner and J. E. Gearien, *J. Pharm. Sci.*, **53**, 1309 (1964).
4. E. Leete, *J. Am. Chem. Soc.*, **84**, 55 (1962).
5. W. J. Griffin, *Naturwissenschaften*, **66**, 58 (1979).
6. E. Leete, *J. Am. Chem. Soc.*, **84**, 55 (1962).
7. D. R. Hoagland and D. I. Arnon, "The Water-Culture Method for Growing Plants without Soil", California Agric. Exp. Station Circular 347 (1950).
8. E. Leete, *Phytochemistry*, **11**, 1713 (1972).
9. W. J. Griffin, H. P. Brand and J. G. Dare, *J. Pharm. Sci.*, **64**, 1921 (1975).
10. G. P. Verzarne, F. Soti and L. Hotavath, *Herba Hung.*, **13**, 77 (1975).
11. K. R. Brain, M. C. Chalopin, T. D. Turner, G. Blunden and P. B. Wildgoose, *Plant Sci. Letters I*, 241 (1973).
12. A. C. Leopold and P. E. Krudermann, "Plant Growth and Development", 2nd Edition, McGraw-Hill, U.S.A., p 249–269.
13. O. Luanratana and W. J. Griffin, *J. Nat. Products*, (in press).

[6]Bell Booth Ltd., Johnsonville, New Zealand.