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HYOSCYAMINE *N*-OXIDE IN *ATROPA BELLADONNA*

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Abstract—Separated organs of *Atropa belladonna* have been examined for their total alkaloid, hyoscyamine and hyoscyamine *N*-oxide contents during ontogenesis. Marked fluctuations in *N*-oxide content were observed, the highest being found in the ripe fruit. [G-^{3}H]-atropine was fed to *A. belladonna* fruits and radioactively labelled hyoscyamine *N*-oxide isolated.

INTRODUCTION

Recently, some members of the Solanaceae have been examined specifically for their alkaloid *N*-oxide content and as a result *N*-oxides of hyoscyamine, hyoscine and nicotine have been isolated as natural products [1,2]. Two isomeric *N*-oxides of hyoscyamine, one having an axial N^+-O^- bond and the other having an equatorial N^+-O^- bond were isolated from several plants, including *A. belladonna* L. [1]. During the initial experiments, it was observed that the yield of *N*-oxide in this plant varied from one organ to another and hence a more detailed investigation of *N*-oxide to tertiary base proportions was undertaken during ontogenesis. In addition [G-^{3}H]-atropine was fed to isolated fruits in order to determine whether *N*-oxidation occurred in the fruits since they proved to have the highest *N*-oxide content.

RESULTS AND DISCUSSION

The roots and the individual aerial parts of *A. belladonna* were collected at six different stages of development, all collections being made at the same time of day. In addition, weekly collections of berries were made over a period of six weeks. Each sample was assayed for the total alkaloid, hyoscyamine and hyoscyamine *N*-oxide contents using a TLC separation for the latter two determinations and measuring the alkaloid colorimetrically by the Vitali–Morin reaction [3–6]. The results are given in Tables 1 and 2 and expressed in terms of percentage hyoscyamine *N*-oxide to hyoscyamine and also in terms of mg hyoscyamine *N*-oxide per 100 g of fresh plant material. The percentage of *N*-oxide to tertiary base ranged from 4.9% in the roots to 34.7% in the seeds at the ripe fruiting stage. In the leaves and in the stems, the percentage of *N*-oxide to tertiary base was at a maximum during the fruiting stages and this corresponded to a decreased percentage in the roots; the fruits proved to have the highest proportion of *N*-oxide for the whole plant. In terms of mg/100 g of fresh weight of plant material, the hyoscyamine *N*-oxide content of the leaves and of the stems reached a maximum at the green fruiting stage but diminished at the ripe fruit stage, whereas in the roots there was a gradual decrease from the young fruit-flowering stage onwards. The actual yield of hyoscyamine *N*-oxide reached its maximum in the ripe fruits so that in terms of percentage *N*-oxide to tertiary base the richest source of *N*-oxide was in the berries, more particularly in the ripe berries.

In order to gain more information about the *N*-oxide variations in the berries, weekly collections were made and assayed. About 1000 flowers with creamy, full grown anthers, were tagged at the same time and this stage of development was termed "week o" (W_o). The fruits were collected at weekly intervals, weighed (*ca* 20 g) and analysed for their total alkaloid, hyoscyamine and hyoscyamine *N*-oxide. The results are given in Table 2. After five weeks ($W_o + 5$), the *N*-oxide reached a maximum of 36 mg/100 g of fresh berry, corresponding to 30% of hyoscyamine *N*-oxide relative to hyoscyamine, but in the subsequent week there was a dramatic fall in the *N*-oxide yield.

To test whether *N*-oxidation occurred in the fruits, [G-^{3}H]-atropine was fed to fruits for four days via their excised stalks. The total alkaloid, hyoscyamine and hyoscyamine *N*-oxide contents were determined for the berries using the method given in the Experimental. Known aliquots of the total alkaloid extract were separated by preparative TLC and the radioactivity of hyoscyamine and its *N*-oxide determined (both compounds were recrystallised to constant specific activity). The results are given in Tables 3 and 4. After four days 25% of the fed radioactivity was located in the berries (Table 3) and of the total radioactivity incorporated in the berries only 30% was found in the alkaloid fraction (Table 4). These results are in agreement with those of Hamon and Youngken [7] who fed tritiated atropine to mature *Datura innoxia* plants and at the end of one day 59% of the introduced radioactivity was found in the non-alkaloidal fraction, whereas after 20 days only 1.5% of the radioactivity resided in the alkaloid fraction. During the present experiment 56.6% of the radioactivity of the total alkaloid was recovered as hyoscyamine but only 2.5% as the *N*-oxide (Table 4). In part, this low activity may be attributed to recrystallisation of the *N*-oxide since of the two isomers present only one could be recrystallised, the other staying in the mother liquor. Furthermore hydrolysis of the ester function in hyoscyamine

Table 1. Total alkaloid, hyoscyamine and hyoscamine *N*-oxide contents of *A. belladonna* at 6 different stages of development

Part of plant and stage of development at time of collection	Collection date (1973)	mg/100 g fr. plant			% *N*-oxide with respect to hyoscyamine
		Total alkaloid	Hyoscyamine	Hyoscyamine *N*-oxide	
A. Before flowering, 7″–10″ tall	7.5				
aerial parts		83.0	70.0	4.9	7.0
roots and root stocks		135.0	110.0	11.0	10.0
B. Initial flowering 2.5′–3′ tall	4.6				
leaves		64.3	55.0	2.8	5.1
stems		108.7	90.0	8.1	9.0
roots and root stocks		123.7	100.0	11.0	11.0
C. Flowering	19.6				
leaves		72.0	60.0	4.8	7.9
stems		74.3	60.0	6.0	10.0
flowering tops		156.2	125.0	15.0	12.0
roots and root stocks		108.7	85.0	12.3	14.5
D. Flowering and young fruits	3.7				
leaves		80.6	65.0	7.8	12.0
stems		68.1	55.0	5.5	10.0
flowering tops		202.5	160.0	24.0	15.0
unripe fruits		143.7	110.0	19.6	17.8
roots and root stocks		103.7	85.0	8.5	10.0
E. Green fruits	18.7				
leaves		96.0	75.0	11.3	15.0
stems		87.5	70.0	9.1	13.0
pulp		125.0	95.0	18.1	19.0
seeds		82.0	60.0	13.8	23.0
roots and root stocks		78.0	65.0	5.2	8.0
F. Blue berries	3.8				
leaves		55.0	40.0	6.8	16.8
stems		46.8	35.0	5.6	15.9
pulp		141.2	100.0	27.0	27.0
seeds		111.2	75.0	26.0	34.7
roots and root stocks		87.0	75.0	3.7	4.9

is one major metabolic route and hence it is possible that radioactivity was lost from the alkaloid fraction by the formation of non-alkaloid metabolites (e.g. tropic acid). The object of the present studies was to try and establish whether hyoscyamine *N*-oxide was formed directly from hyoscyamine in *A. belladonna* fruits and despite the inherent difficulties in making conclusions from using only ^3H precursors, the results do lead to the not unreasonable suggestion that hyoscyamine itself undergoes *N*-oxidation. This does not rule out the possibility that tropine formed by hyoscyamine hydrolysis undergoes *N*-oxidation and re-esterification with tropic acid to yield hyoscyamine *N*-oxide. Although both these possibilities exist the former must be considered to be the more probable, but whichever pathway is involved the results do indicate that some *N*-oxidation occurs in the fruits. The marked decrease in the *N*-oxide content of the roots during the flowering and fruiting stages (Table 1) points to the further possibility of *N*-oxide being transferred to the developing fruit. Control experiments clearly indicate that *N*-oxidation does not occur during isolation procedures [1].

Table 2. Total alkaloid, hyoscyamine and hyoscyamine *N*-oxide contents of *A. belladonna* fruits collected at weekly intervals

Collection date (1974)	Fruit age*	mg/100 fr. plant			% *N*-oxide with respect to hyoscyamine
		Total alkaloid	Hyoscyamine	Hyoscyamine *N*-oxide	
20.6	Wo	110.0	80.0	10.0	12.5
27.6	Wo + 1	130.0	97.5	15.0	15.4
4.7	Wo + 2	125.0	91.3	14.0	15.3
11.7	Wo + 3	146.0	103.7	20.0	19.3
18.7	Wo + 4	180.0	130.0	35.0	26.9
25.7	Wo + 5	170.0	120.0	36.0	30.0
1.8	Wo + 6	128.0	90.0	18.0	20.0

* Collections made at weekly intervals; Wo = time at which flowers had fully grown creamy anthers.

Table 3. Distribution of radioactivity after feeding [G-^{3}H]-atropine (43.96 × 10^6 dpm) to 180 isolated *A. belladonna* fruits

	Radioactivity (dpm × 10^6)	% Radioactivity of total fed
5% NH_4OH–MeOH extract of:		
stalks (15.83 g)	21.41	48.7
fruits (172.0 g)	11.19	25.4
residual activity in tubes	10.34	23.5

As previously indicated, the role of these *N*-oxides is not understood although they may play a part in *N*-demethylation since they readily decompose thermally to the corresponding nor-alkaloids [1,2]. A further speculation is in the biosynthesis of hyoscine since a Dreiding model of the hyoscyamine *N*-oxide isomer which has the equatorial N^+-O^- bond shows that the oxygen is close to the C-6 and C-7 β hydrogens and hence in a similar position to the epoxide oxygen in hyoscine. Currently it is beleived that hyoscine may be formed from hyoscyamine via 6-hydroxyhyoscyamine [8,9].

The present results clearly demonstrate that marked variation in hyoscyamine *N*-oxide content occurs during ontogenesis and if this is related to the fact that hyoscyamine is rapidly metabolised in the plant then it may be assumed that the *N*-oxide plays an important role in hyoscyamine metabolism. Certainly the increased polarity of the *N*-oxide compared with that of the tertiary base cannot be ignored and indeed these differences may be significant in the retention or exclusion of hyoscyamine from cells or organelles, or in general transport mechanisms.

EXPERIMENTAL

The plants examined were 2nd yr plants grown at the Experimental Garden Myddelton House, Enfield, Middlesex; all collections were made at 15.00 hr. For the weekly examination of alkaloids in the fruits, 1000 flowers with creamy full-grown anthers were tagged at the same time (termed week o). Approximately 20 g of fruit were collected each week for six weeks. Berries from 1st yr plants were used for the radioactive feeding experiments.

Extraction of alkaloids. The plant material was extracted in a blender with 5% NH_4OH in MeOH and maceration continued for 18 hr. The filtered extract was concentrated under reduced pressure to a semi-solid which was extracted into 2% H_2SO_4. The filtered acid extract was made alkaline with dil. NH_4OH and extracted successively with $CHCl_3$ and $CHCl_3$–NH_4OH (9:1) and the combined extracts evaporated to dryness.

Estimation of alkaloid and N*-oxide content.* A known wt of fr. plant material (20 g in the case of seeds and fruits; 40 g in all other cases) was extracted as described above and total alkaloid residue dissolved in $CHCl_3$–MeOH (1:1, 5 ml). Aliquots of this extract were used for the estimation of total alkaloid, of hyoscyamine and of its *N*-oxide using the Vitali–Morin reaction [6]. A 0.02 ml aliquot of the total alkaloid extracted was evaporated carefully to dryness on a steam bath and the residue was nitrated by adding fuming nitric acid (1 ml) which was subsequently removed by evaporation. On cooling, the residue was dissolved in DMF, 25% tetraethylammonium hydroxide (0.5 ml) added and the vol made up to 10 ml with DMF. After 5 min colour density was measured at 540 nm and total alkaloid content calculated as hyoscamine using a hyoscyamine calibration curve. For estimation of hyoscyamine and hyoscyamine *N*-oxide, aliquots of the alkaloidal solution (1 ml and 3 ml respectively) were subjected to preparative TLC using Sil gel G with A. Me_2CO–H_2O–conc. NH_4OH (90:7:3) for hyoscyamine and B. EtOAc–*iso*-PrOH–20% NH_4OH (45:35:15) for hyoscyamine *N*-oxide. Elution of the alkaloids from the Si gel was with $CHCl_3$–EtOH (1:1). The eluates were evaporated to dryness and the residues dissolved in known volumes of $CHCl_3$–MeOH (1:1). and subjected to the Vitali–Morin reaction as described above. The concentration of hyoscyamine and its *N*-oxide were determined from their respective calibration curves. The results are given in Tables 1 and 2.

Administration of [G-^{3}*H*]*-atropine* (Radio-Chemical Centre, Amersham, Bucks). 180 green fruits of *A. belladonna* were excised and the cut stalks placed in Eppendorf tubes containing dist. H_2O. Prior to feeding, most of the H_2O was removed and [G-^{3}H]-atropine (sp. act. 1.52 m Ci/mg) soln in dist. H_2O (0.2 m., 244 × 10^3 dpm) added to each tube. Further distilled H_2O was added as required for 4 days.

Determination of alkaloid radioactivity. The stalks were washed with H_2O and separated from the fruits and each extracted separately using the method given above (fruits 172 g, stalks 15.83 g). The radioactivity of the total 5% NH_4OH/MeOH extracts, the total alkaloid, hyoscyamine and hyoscyamine *N*-oxide (the latter two being separated by prep. TLC as described above) were determined from phosphor solutions (15 ml) using a Packard Tricarb 3003 liquid scintillation counter. The total alkaloid, hyoscyamine and hyoscyamine *N*-oxide were estimated by the Vitali–Morin reaction as described above and the results are given in Tables 3 and 4. From one-fifth of the total alkaloid solution of the fruits, hyoscyamine and hyoscyamine *N*-oxides were isolated by prep. TLC (systems A. and B.). To the radioactive *N*-oxide (7.6 mg, mixed isomers) was added cold *N*-oxide (50 mg, equatorial N^+–O^- bond isomers) and to the radioactive hyoscyamine (40 mg) was added cold hyoscyamine (50 mg) for recrystallisation. After two recrystallisations from EtOH–Et_2O, hyoscyamine hydrochloride was found to have a sp. act. of 3610 dpm/mg and hyoscyamine *N*-oxide hydrochloride 184 dpm/mg.

Table 4. Radioactivity of alkaloids in *A. belladonna* fruits (172 g, total MeOH extract 11.19 × 10^6 dpm)

Fraction	Mg alkaloid (calc. as hyoscyamine from Vitali)	Radioactivity (dpm × 10^6)	Specific activity (dpm/mM × 10^6)	Recovered activity as % of MeOH extract
Total alkaloids	275	3.39	—	30.30
Hyoscyamine	200	1.92 (56.6%)*	2.78	17.16
Hyoscyamine *N*-oxide	38	0.086 (2.5%)*	0.69	0.77

* Figures represent recovered activity as % of total alkaloid fraction.

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