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LIMITATIONS OF FEEDING EXPERIMENTS IN STUDYING ALKALOID BIOSYNTHESIS IN *PEGANUM HARMALA* CALLUS CULTURES

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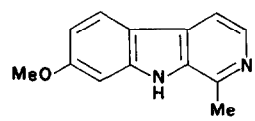
Key Word Index—*Peganum harmala*; Zygophyllaceae; biosynthesis; alkaloids; β-carbolines; callus cultures; compartmentation.

Abstract—Feeding and trapping experiments to *Peganum harmala* callus cultures were limited by compartmentation; exogenous substrates were detoxified by precipitation, presumably as polymers or conjugates, or by conversion to water-soluble products, such as phenols and glucosides, easily stored in vacuoles. Alkaloid-producing and non-alkaloid-producing callus cultures were readily able to convert tryptamine to 5-hydroxytryptamine and harmaline to dihydroruine (8-hydroxyglucosylharmaline). Phenolic substrates, including 5- and 6-hydroxytryptophan, 5- and 6-hydroxytryptamine and harmalol, were not metabolized. In alkaloid-producing callus cultures, radioactivity from [methylene-^{14}C]-L-tryptophan and [methyl-^{14}C-]-harmaline was incorporated into harmine. The dilution of radioactivity was 30000- and 2-fold respectively.

INTRODUCTION

Peganum harmala has long been known as a source of β-carboline alkaloids. Their synthesis from tryptophan has been shown in sterile roots[1] and confirmed in seedlings by Stolle and Groeger,[2] who also showed that harmine (**1**) would incorporate the β-C and β-N atoms from tryptamine into the pyridine ring, the methyl group from methionine into the methoxy group, and the pyruvate C-2 and C-3 atoms into the C-1 and C-methyl positions. From these results they postulated that 1,2,3,4-tetrahydroharman-1-carboxylate, formed by the condensation of tryptamine with pyruvate might be an intermediate. This could not be confirmed by direct feeding.



(**1**)

These results also indicate that the hydroxyl group is introduced *after* decarboxylation, as in the synthesis of bufotenin in *Piptadenia peregrina*,[3] psilocybin by *Psilocybe*[4] and serotonin in tomato and barley shoots,[5] rather than before, as in serotonin synthesis by animals and 5-hydroxytryptophan synthesis in *Griffonia simplicifolia*.[6] The mechanism of cyc-

[1] GROEGER, D. and SIMON, H. (1963) *Abhand. Deut. Akad. Wiss., Kl. Chem. Geol. Biol.* **4**, 343.
[2] STOLLE, K. and GROEGER, D. (1968) *Arch. Pharm.* **301**, 561.
[3] FELLOWS, L. E. and BELL, E. A. (1971) *Phytochemistry* **10**, 2083.
[4] AGURELL, S. and NILSSON, J. G. (1968) *Acta Chem. Scand.* **22**, 1210.
[5] WIGHTMAN, F., GIBSON, R. A. and SCHNEIDER, E. A. (1970) *7th Int. Conf. on Plant Growth Substances*, Canberra, Australia, Abstract 106.
[6] FELLOWS, L. E. and BELL, E. A. (1970) *Phytochemistry* **9**, 2389.

lization of the tryptamine derivative to the harmala alkaloids is unknown; it is discussed by Slaytor and McFarlane[7] who found that harman in *Passiflora edulis* is probably derived via *N*-acetyltryptamine and harmalan.

In this paper we report the results of feeding and trapping experiments designed to help elucidate the biosynthesis of the β-carbolines in root callus cultures from *P. harmala*. These experiments were carried out with likely precursors which were radioactively labelled. The effects on growth and differentiation of the callus using unlabelled substrates was also noted.

RESULTS

Feeding experiments with tryptophan

Non-radioactive tryptophan was not appreciably converted to EtOH-soluble products. On SM medium (sucrose-minerals)[8] traces of tryptamine and 5-hydroxytryptamine could be detected. Tyrptophan on SM medium stimulated differentiation.

TABLE 1. DISTRIBUTION OF RADIOACTIVITY AFTER FEEDING ^{14}C-TRYPTOPHAN TO *Peganum harmala* CALLUS

Callus grown on	dpm
SMD	
EtOH-insoluble residue	500000
80% EtOH extract	82000
SM	
EtOH-insoluble residue	120000
80%EtOH extract	110000
Hydroxytryptamine region of TLC	40000
Harmine region of TLC (= 50 nmol harmine)	400
Harmine recrystallized to constant sp. act.*	200

* 25·4 mg carrier harmine was added to 22 nmol eluted from TLC. 1st recrystallization (ex MeOH) (dpm/mg), 3·2; 2nd recrystallization (ex MeOH-EtOAc), 3·0; 3rd recrystallization, 3·4. Sp. act. 1·7 μCi/mM.

These results were studied in detail with ^{14}C-tryptophan. Autoradiograms of TLCs of the EtOH extract from callus grown on SMD medium (sucrose-minerals-2,4-D)[9] showed that tryptophan was the only significant radioactive compound. Tryptophan and 5-hydroxytryptamine were the major radioactive compounds in extracts from callus grown on SM (Table 1); very little activity was present in the β-carbolines. Most radioactivity in the calluses, especially from that on SMD, was insoluble in EtOH; presumably it was in protein or conjugates. The harmine was not degraded to localize the incorporated label which accounted for 0·1% of the activity in the callus, the activity being diluted 30000-fold. This dilution does not exclude an indirect route for incorporation.

Tables 2 and 3 present the results of attempted trapping experiments using an unlabelled trap (1 μmol) and ^{14}C-tryptophan (30 nmol). In every case in Table 2 autoradiograms showed a very small amount of label was incorporated into the harmalol, harmaline and

[7] SLAYTOR, M. and MCFARLANE, I. J. (1968) *Phytochemistry* **7**, 605.
[8] NETTLESHIP, L., MCKENZIE, E. and SLAYTOR, M. (1974) *J. exp. Bot.*, submitted for publication.
[9] NETTLESHIP, L. and SLAYTOR, M. (1974) *J. exp. Bot.*, submitted for publication.

harmine. The bulk of radioactiviy in the EtOH extracts was present as unchanged tryptophan and as 5-hydroxytryptamine. As 5- and 6-hydroxytryptamine co-crystallised, careful co-chromatography was necessary to confirm that 6-hydroxytryptamine contained little label and that 5-hydroxytryptamine was highly radioactive. At least 10% of the exogenous tryptophan was converted to 5-hydroxytryptamine. None of the substrates used as traps

TABLE 2. RADIOACTIVITY IN HARMINE AND HARMALOL FROM CALLUS GROWN IN THE PRESENCE OF A TRAP AND ^{14}C-TRYPTOPHAN

Potential trap	Harmalol (nmol)	Harmine (nmol)	Harmalol region of TLC (dpm × 10^{-3} in)	Harmine region of TLC (dpm × 10^{-3} in)
None	140	350	9·0	7·9
5-Hydroxytryptophan	230	480	13·4	13·6
6-Hydroxytryptophan	150	260	10·8	8·2
5-Hydroxytryptamine creatinine.SO_4	290	530	6·1	6·2
6-Hydroxytryptamine creatinine.SO_4	240	420	10·0	9·2
6-Methoxytryptamine.HCl	170	340	5·0	7·0
Harmalol.HCl	130	230	12·4	7·6
Harmaline.HCl	230	540	12·8	13·6
6-Methoxyharmalan.HCl	180	370	8·8	7·1
Harmol.HCl	130	270	8·4	6·8

TABLE 3. COMPOUNDS FROM TRAPPING EXPERIMENTS RECRYSTALLIZED TO CONSTANT SPECIFIC ACTIVITY

Compound	dpm × 10^{-3} (TLC)	dpm × 10^{-3} (Constant sp. act.)	Carrier added (mg)	Recrystallisation solvent
5-Hydroxytryptamine from callus fed 5-hydroxytryptamine	85[1]	36	105[5]	H_2O–acetone
6-Hydroxytryptamine from callus fed 6-hydroxytryptamine	Not assayed[2]	Nil	100[5]	H_2O–acetone
Harmalol: from callus fed harmaline	13[2]	3·6	60[4]	MeOH–EtOEt
Harmaline: from callus fed harmaline	1·4[2]	0·5	60[4]	MeOH–EtOEt
6-Methoxyharmalan from callus fed 6-methoxyharmalan	0·6[2]	Nil	32[3]	MeOH–EtOAc
6-Methoxytryptamine from callus fed	Not			
6-Methoxytryptamine from callus fed 6-methoxytryptamine	Not assayed[2]	Nil	19[3]	C_6H_6

Each compound was eluted from the TLC of that extract which contained the most of it, usually from the callus to which it was fed as trap. 1/10[1] or 9/10[2] of it was combined with non-radioactive carrier and recrystallized to constant sp. act. as the free base,[3] or the hydrochloride[4] or the creatinine SO_4 complex. The drop in radioactivity from the second to the third column is due to smearing of radioactive impurities over the TLC and to decomposition of the compound between TLC and recrystallization.

blocked incorporation into harmine and harmalol. This is probably due to translocation and compartmentation problems, and to formation of β-carbolines before addition of the potential traps. However, the substrates did help to protect and to identify endogenously formed intermediates (Table 3). Only traces of hydroxytryptophans could be detected on TLC of EtOH extracts, although they were probably taken up, as tryptophan was. Whether their disappearance is for artefactual or metabolic reasons it could account for the lack of incorporation of radioactivity in the hydroxytryptophan region of TLC's after feeding ^{14}C-tryptophan. Thus this experiment does not confirm or deny that 6-hydroxytryptophan is an intermediate in β-carboline synthesis.

The ratio of the dpm in harmalol and harmaline recrystallized to constant sp. act. approximated their relative molar concentrations. This indicates they are intermediates in the biosynthesis of harmine; the percentage incorporation from tryptophan is very low and the dilution of sp. act. high: for harmalol, 0·2% and 6000-fold respectively. No radioactivity was incorporated into 6-methoxytryptamine or 6-methoxyharmalan; but since, like other substrates, they probably did not penetrate to the site of alkaloid synthesis, this does not prove that they cannot be formed from endogenous tryptophan. The possibility remains that 6-methoxytryptamine may be an intermediate present in vanishingly small amounts because of a rapid conversion to a β-carboline alkaloid, e.g. harmaline.

TABLE 4. DISTRIBUTION OF RADIOACTIVITY AFTER FEEDING ^{14}C-TRYPTAMINE TO *Peganum harmala* CALLUS

Callus grown on	dpm
SMD	
EtOH-insoluble residue	23400
80%EtOH extract	69400
SM	
EtOH-insoluble residue	27400
80% EtOH extract	77200
5-Hydroxytryptamine region of TLC	50000
Harmine region, first TLC	1500
Harmine region, second TLC	500
Recrystallization of harmine:	
1st recrystallization, from MeOH: per mg	<0·1

Harmine from the first TLC was eluted and rerun; 50 nmol eluted from second TLC was combined with 45 mg carrier for recrystallization.

Feeding experiments with tryptamine

Non-radioactive tryptamine produced much 5-hydroxytryptamine on both media and may have slightly lowered growth. 5-Hydroxytryptamine was isolated in 32% yield from callus grown on SMD for 4 weeks. On SM medium tryptamine stimulated differentiation.

The results of feeding [α^{14}C]tryptamine are in Table 4. Autoradiograms of TLCs of EtOH extracts from SMD- and SM-grown callus showed most of the radioactivity was in 5-hydroxytryptamine; little remained as tryptamine, and none was incorporated in β-carbolines or indole acetic acid. Radioactivity in the harmine region of the TLCs illustrates the smearing of impurities (here from decomposing hydroxytryptamine) over preparative plates.

Feeding experiments with harmaline

Large amounts of dihydroruine (8-hydroxyglucosylharmaline),[8] as well as ruine (8-hydroxyglucosylharmine)[10] and harmine were extracted from callus grown on SM and SMD media to which harmaline had been added. For example, harmaline. HCl, cold sterilized onto SMD medium was converted by the callus in 2 weeks to dihydroruine and isolated in 21% yield. Similarly, ^{14}C-harmaline was converted to dihydroruine, ruine and harmine (Table 5).

TABLE 5. INCORPORATION OF RADIOACTIVITY FROM HARMALINE INTO EtOH-INSOLUBLE MATERIAL, HARMINE, RUINE AND DIHYDRORUINE

	Callus grown on SMD			Callus grown on SM		
	nmol	dpm	Sp. act. μCi/mM	nmol	dpm	Sp. act. μCi/mM
EtOH-insoluble residue		4500			8800	
80% EtOH extract		11000			16000	
Harmaline	120	2900*	11*	110	2800*	12*
Dihydroruine	140	4000*	13*	140	4100*	13*
Harmine	Trace			90	1600* 1300†	8·1* 6·5†
Ruine	Trace	100*		120	2700*	10*
Harmalol	Nil			40	Nil	
Harmine from medium	Trace			130	2700* 2500‡	9·5* 8·5‡

* From dpm in respective TLC regions.
† After recrystallization to constant sp. act. (ex MeOH) 47 mg carrier added to 40 nmol from callus.
‡ After recrystallization to constant sp. act. (ex MeOH) 51 mg carrier added to 100 nmol from medium.

Autoradiograms of TLCs of EtOH extracts of callus from both media show the major product is dihydroruine. Only traces of radioactive harmine were found in the SMD medium and the callus grown on it. There were larger amounts of ruine. The total content of harmine + ruine (about 3% of harmaline fed, based on radioactivity) can be easily accounted for by autooxidation of harmaline. However, much label was incorporated into harmine and ruine in callus on SM. The medium itself contained appreciable amounts of harmine, but not ruine. Harmalol was not labelled indicating there was no demethylation of the harmaline. Thus callus on SM can convert harmaline to harmine; but not necessarily at the site of endogenous alkaloid synthesis. Though the endogenous pool of harmaline is normally small, expected to be found 4 nmol in this experiment, and the amount of exogenous harmaline taken up is comparatively large, over 300 nmol in the SM callus, there is nearly 50% dilution of the sp. act. of the harmine formed; and since sp. act. harmaline fed > sp. act. harmine from medium > sp. act. harmine in callus, it appears that some harmine has been excreted into the medium. Normally, no alkaloids are excreted. These results, together with the high rate of formation of dihydroruine, point to restricted intermixing between pools of harmaline formed endogenously and taken up from exogenously supplied alkaloid.

Feeding experiments with phenolic substrates

5- and 6-hydroxytryptophan were not appreciably converted to EtOH-soluble products; 5- and 6-hydroxytryptamine may have slightly lowered growth. 6-Hydroxytryptophan and

[10] NETTLESHIP, L. and SLAYTOR, M. (1971) *Phytochemistry* **10**, 231.

6-hydroxytryptamine were unstable; they rapidly decomposed under these feeding conditions whether or not callus was present on the medium. 5-Hydroxytryptophan and 5-hydroxytryptamine were much less labile. 6-Hydroxytryptamine inhibited growth on SMD and produced harmalol-like artefacts on both media. Creatinine, present in the substrate complexes 5- and 6-hydroxytryptamine creatinine sulphates had no effect on metabolism at a concentration of 0·2 mM. Harmalol and harmol, though taken up by callus, remained unchanged.

Feeding experiments with 5-methyltryptamine, 6-methoxytryptamine and harman

5-Methyltryptamine was converted to an unidentified phenolic derivative based on colour reactions on TLC.[8] 6-Methoxytryptamine produced alkaloid-like artefacts on both media. The artefacts had fluorescence and TLC properties similar to harmaline and harmine. Harman, though remaining largely unaltered, appeared to be converted to a ruine analogue.

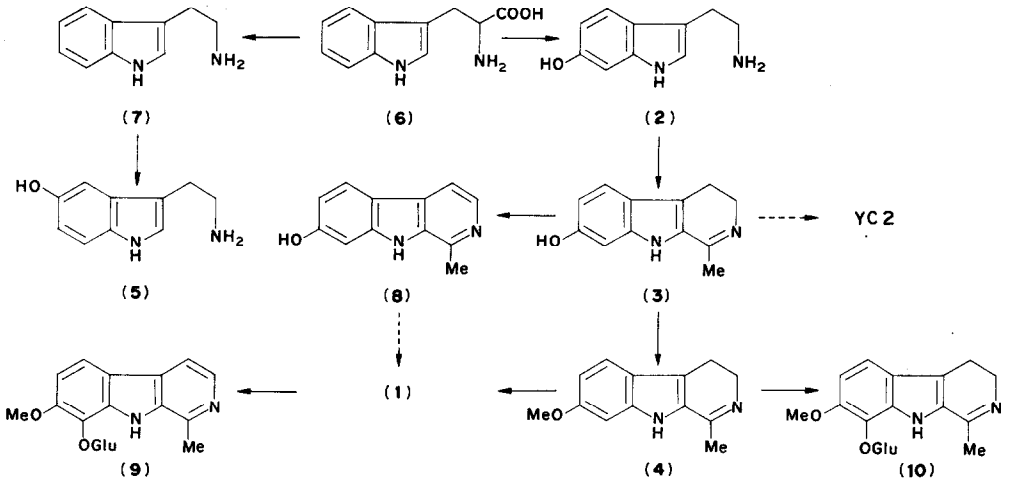
DISCUSSION

Peganum harmala callus grown on a medium referred to as SMD medium,[9] containing the auxin 2,4-dichlorophenoxyacetic acid does not produce β-carboline alkaloids while callus grown on a medium, referred to as SM medium,[8] containing no auxin produces alkaloids. The initiation of alkaloid synthesis when callus is transferred from SMD medium and to SM medium requires the synthesis of specific enzymes.[8] The failure to demonstrate the incorporation of any precursors into harmine in callus grown on SMD medium is consistent with this. Although callus grown on SMD medium cannot synthesize alkaloids from exogenous substrates it can readily detoxify several potential precursors by non-specific hydroxylation or hydroxylation and glucosylation. Thus tryptamine is converted to 5-hydroxytryptamine and harmaline and harmine are converted to their 8-hydroxyglucosyl derivatives, dihydroruine and ruine,[10] respectively. These metabolites are all found in the whole plant where presumably they are similarly formed. Callus grown on SMD medium can detoxify other substrates which are not normally found in the plant. 6-Methoxyharmalan and harman apparently can be converted to hydroxyglycosides, while 5-methyltryptamine is hydroxylated. The reaction of 5-methyltryptamine is significant as it implies that this hydroxylation is not necessarily in the 5-position. Phenolic β-carbolines, hydroxytryptamines and hydroxytryptophans are not apparently metabolized this way when fed. There is no evidence that they or their endogenously formed counterparts are directly glucosylated.

Alkaloid-producing callus grown on SM medium is also capable of carrying out these reactions but in addition it can effect the synthesis of harmine from exogenous ^{14}C-tryptophan and ^{14}C-harmaline. It seems that only harmaline, out of the possible intermediates tried, really reached the site of alkaloid synthesis and then only partially. In general, feeding experiments were interfered with by compartmentation.[11] Other intermediates were neutralized by hydroxylations and glycosylations which confer extra solubility in the vacuolar sap, or presumably polymerizations and conjugations which precipitate them at the boundary of the cell. These observations are similar to those of Innerarity *et al.*[12] who fed scopoletin to tobacco seedlings. The limitations of the feeding experiments affected the

[11] OAKS, A. and BIDWELL, R. G. S. (1970) *Ann. Rev. Plant Physiol.* **21**, 43.
[12] INNERARITY, L. T., SMITH, E. C. and WENDER, S. H. (1972) *Phytochemistry* **11**, 1389.

usefulness of trapping experiments. No trap was able to stop harmine synthesis from ^{14}C-tryptophan and the only intermediates which accumulated any label were harmalol and harmaline. The incorporation of substrates by *P. harmala* callus into compounds known to occur in the whole plant is consistent with Fig. 1. This Figure is also in agreement with the dynamics of formation of the alkaloids.[8]



⇢ possible steps → probable steps

SCHEME 1. BIOSYNTHESIS AND METABOLISM OF ALKALOIDS IN *Peganum harmala* CALLUS.

This model agrees with the literature insofar as it postulates that intermediates in the biosynthesis of harmine are 6-hydroxytryptamine[13] (**2**) and harmalol (**3**) and harmaline[1] (**4**); and that 5-hydroxytryptamine (**5**) is biosynthesized from tryptophan (**6**) via tryptamine[5] (**7**). The substrate of the first hydroxylation could not be determined, primarily because of compartmentation difficulties. 6-Hydroxytryptamine was not definitely shown to be an intermediate; its presence[8] does not provide conclusive evidence, especially as other strains of *P. harmala* apparently do not contain detectable amounts of it. Build-up of it may be a strain specific side-reaction, like the accumulation of 5-hydroxytryptamine. In view of the compartmentation problems, the lack of incorporation of tryptamine into harmine does not exclude its role as an intermediate as shown by Stolle and Groeger.[2] Rather, it points to a difference in transport systems between the callus and whole roots. The possible conversion of harmol (**8**) to harmine (**1**) was not checked but it is not unlikely as methylations are often non-specific.[14] Speculatively, YC2, an unidentified phenolic dihydro-β-carboline,[8] may be a hydroxyglycosylated derivative from harmalol.

EXPERIMENTAL

General. All reagents and solvents were of analytical grade or were purified before use. The following were purchased: [methylene-^{14}C]L-tryptophan (sp. act. 54·6 mCi/mM) from the Radiochemical Centre, Amersham; 6-methoxyindole and 6-methoxyharmalan from Regis Chemical Co., Chicago, U.S.A.; *N*-methyltryptamine and 5-methyltryptamine. HCl from Sigma Chemical Co., St. Louis, U.S.A. The following were synthesized: 6-methoxy-

[13] DAGLEISH, C. E. (1961) *Arch. Biochem. Biophys.* **94,** 543.

[14] SMITH, J. N. (1964) in *Comparative Biochemistry* (FLORKIN, M. and MASON, H. S., eds.), Vol. VI, p. 403, Academic Press, New York.

tryptamine (from 6-methoxyindole via the gramine derivative) [α-^{14}C]tryptamine (sp. act. 0·16 mCi/nmol)[15] and [methyl-^{14}C]harmaline (sp. act. 12 μCi/mM). The ^{14}C-harmaline was synthesized from 6-methoxytryptamine and [methyl-^{14}C]acetic anhydride. Other chemicals and biochemicals were obtained as described previously.[8,9]

Feeding experiments with unlabelled substrates. Callus was grown for 21 days under conditions described previously on SM[8] and SMD[9] media. In each experiment 1 μmol substrate per 10 ml medium was used and the substrate was cold sterilized onto the medium. The experiments were repeated using 10 μmol substrate. Large scale feeding experiments with tryptamine and harmaline for the isolation of 5-hydroxytryptamine and dihydroruine respectively were each carried out using 500 ml SMD medium in 10 × 150 ml flasks. The substrate was cold sterilized onto the medium to give a final concentration of 1 μmol/ml harmalol.HCl, harmaline.HCl and harmol.HBr.

Feeding experiments with radioactive substrates. Stock 1 callus (10 ml) was grown for 21 days on SM and SMD media. The radioactive substrates were either cold-sterilized onto the medium (tryptophan and tryptamine) or autoclaved with the medium (harmaline). The following amounts of radioactive substrates were used: [methylene-^{14}C]L-tryptophan (7 nmol; 8·8 × 10^5 dpm); [α-^{14}C]tryptamine (1 nmol; 3·6 × 10^5 dpm); [methyl-^{14}C]harmaline (2·3 μmol; 6 × 10^4 dpm).

Feeding experiments with ^{14}C-tryptophan and unlabelled traps. Stock 1 callus was grown for 19 days on SM medium. After 13 days, 1 μmol of one of the unlabelled traps was cold-sterilized onto each 10 ml medium. The unlabelled traps were 5- and 6-hydroxytryptophan, 5- and 6-hydroxytryptamine creatinine. SO_4, 6-methoxytryptamine.HCl, harmalol.HCl, harmaline.HCl, 6-methoxyharmalan.HCl and harmol.HCl. After 1 more day [methylene-^{14}C]L-tryptophan (3 × 10^6 dpm; 30 nmol) was cold sterilized into each tube.

Estimation of alkaloids. Alkaloids were extracted from the callus, separated by TLC, identified and estimated as described previously.[8]

Radioactive estimations. Autoradiograms were prepared using Kodirex Medical X-ray film [Kodak (Australasia) Pty. Ltd.]. Packard Tri-Carb Liquid Scintillation Spectrometers Models 2202 and 3375 were used to measure the radioactivity in vials containing samples in 10 ml scintillant (4g 2,5-phenyloxazole (PPO) and 100 mg 1,4-bis-2-(4-methyl-5-phenyl-oxazolyl) benzene (DiMePOPOP) from Packard per l. toluene). When possible, samples were solubilized with the minimum amount of MeOH of Packard Soluene. Soluene quenched less than MeOH, but caused decomposition of phenolic bases to brown products; thus MeOH was preferable as a solubilizer for harmalol and the hydroxytryptamines. Intensely coloured solutions could sometimes be bleached with benzoyl peroxide. TLC scrapings and EtOH-insoluble residues were counted as suspensions, using 0·3 g Packard Thixotropic Gel Powder per vial. Since all counts were internally standardized with ^{14}C-toluene they are expressed as (dpm).

Acknowledgements—This work was supported by the Australian Research Grants Committee and by the award of a Commonwealth Postgraduate Research Award to one of us (L.N.).

[15] BAXTER, C. and SLAYTOR, M. (1972) *Phytochemistry* **11**, 2767.