Enhanced 3-methoxytyramine levels in crown gall tumours and other undifferentiated plant tissues

Stephen D. MITCHELL,*† John L. FIRMIN† and DAVID O. GRAY*
*Department of Botany and Biochemistry, Westfield College, Hampstead, London NW3 7ST, U.K., and
†John Innes Institute, Colney Lane, Norwich NR4 7UH, U.K.

(Received 9 May 1984/ Accepted 11 June 1984)

An amine, after dansylation, has been isolated from *Nicotiana tabacum* crown gall tumours for the first time and characterized as 4-hydroxy-3-methoxy-β-phenylethylamine (3-methoxytyramine). The compound cannot be detected in differentiated *N. tabacum* tissues but appears in the corresponding callus controls. Its concentration is further increased 5-42-fold when *N. tabacum* is transformed with all strains of *Agrobacterium tumefaciens* so far tested.

When dicotyledenous plants are inoculated with *Agrobacterium tumefaciens*, control of cell division is disrupted by incorporation of a large tumour-inducing (Ti) bacterial plasmid into the eukaryotic cell (Van Larabeeke, 1974; Chilton et al., 1977). The resulting crown gall, which normally becomes sterile because the *Agrobacterium* plays no further part in its maintenance, normally grows rapidly as an undifferentiated mass comparable with many animal tumours (Drummond, 1979). Crown galls are well known for the production of opines, abnormal amino acids determined by the Ti plasmid and serving as specific bacterial substrates (Goldman et al., 1968). Despite this, there has only been one previous investigation of the corresponding amine fractions, limited to the measurement of polyanyme levels (Bagni et al., 1972). A wider ranging investigation intended to identify crown gall amines that may function as opines has now shown the presence of 4-hydroxy-3-methoxy-β-phenylethylamine (3-methoxytyramine) and an apparent correlation between its concentration and tissue morphology.

**Experimental**

**Materials**

[^14C]Tyrosine was purchased from The Radiochemical Centre, Amersham. CM52 (a grade of carboxymethylcellulose) was supplied by Whatman Biochemicals. Dansyl chloride and 3-methoxytyramine were obtained from Sigma. Silica gel, the polyamide layers and all other chemicals came from BDH.

**Culture of tumours**

Seedlings inoculated with *A. tumefaciens* were grown from surface-sterilized seeds germinated in the presence of antibiotics. Fragments of the tumours produced were grown up on an agar medium, initially containing antibiotics, and were periodically tested for bacterial contamination. Untransformed (control) callus tissue was grown on the same medium supplemented with 1-naphthylacetic acid (2mg/l) and 6-benzylaminopurine (25μg/l). The techniques have been detailed as 'method 2' by Butcher et al. (1980). Here the tumour material has been specified by giving the host plant first, followed by the strain of *A. tumefaciens* used to transform it.

**Determination of 3-methoxytyramine**

Exposure of dansylated amines to light was avoided and all operations were carried out at 20–25°C unless otherwise stated. Tissue (10g fresh wt.) was homogenized with 30ml of methanol and, after filtration, this extract was evaporated to dryness in vacuo at 50°C. The residue was dissolved in 150ml of water and 20% (≡2g fresh wt.) was applied to columns (10cm x 1cm) of CM52. The CM52 was in the Na+ form as supplied after removal of fines. Each column was washed with 100ml of water and the amine fraction was eluted with 100ml of 0.5M-HCl. Each eluate was evaporated to dryness as before and excess HCl was removed by redissolving the residue in 10ml of water and re-evaporating, normally twice.
Derivatization and chromatography was a modified form of the Seiler & Wiechmann (1970) procedure. Thus the amine fraction in 1 ml of water was vortex-mixed with 1 ml of dansyl chloride (3 mg/ml in acetone) and 2 mg of Na₂CO₃ for 1 min and left for 18 h in darkness. After adding 3 mg of proline and leaving a further 2 h, the dansylated amines were extracted with 2 × 5 ml of ethyl acetate.

The ethyl acetate was evaporated to dryness under a stream of air at 50°C and the residue was redissolved in 50 µl of ethyl acetate before applying it all to a 0.25 mm thick, 20 cm × 20 cm layer of silica gel 60G. This was chromatographed in the first direction in solvent 1 [cyclohexane/ethyl acetate (2:3, v/v)] and the second in solvent 2 [benzene/triethylamine (5:1, v/v)]. The appropriate complex of spots, located under u.v. light (350 nm), was extracted with 5 ml of acetone/water (9:1, v/v). The product was evaporated and rechromatographed as before but this time one-dimensionally in solvent 3 [chloroform/butyl acetate (8:3, v/v)].

All spots co-chromatographing with mono- and bis-dansyl-3-methoxytyramine were extracted with 1 ml of acetone (SpectrosoL grade) and their fluorescence was measured (excitation, 358 nm; emission, 485 nm). The derivatives recovered from duplicate samples of each tissue type were estimated by comparison with standards (0–100 µg) after allowing for the inherent fluorescence of silica gel blanks.

Small scale isolation of monodansyl-3-methoxytyramine

The procedure was similar to that already described except that the amine fraction from 10 g fresh wt. of N. tabacum/B0542 tumour material was isolated with a column (10 cm × 1 cm) of CM52 in the H⁺ form.

After derivatization and two-dimensional t.l.c. as before, the eluted sample was redissolved in 10 µl of acetone (ArlaR grade) and finally purified by chromatography in benzene/acetic acid (9:1, v/v) on a 15 cm × 15 cm polyamide layer prewashed in methanol/acetic acid (3:1, v/v). This enabled the very small sample of monodansyl-3-methoxytyramine isolated (about 0.15 µg) to be introduced into a mass spectrometer more efficiently than usual, while still absorbed to polyamide particles as recommended by Kraft et al. (1981). The experimental spectrum was corrected for that given by a polyamide blank.

Larger scale isolation of monodansyl-3-methoxytyramine

Details unspecified are the same as in the section ‘Determination of 3-methoxytyramine’. N. tabacum/ACH5 tumour material (100 g fresh wt.) was homogenized with 300 ml of methanol; the extract was filtered and evaporated as before. The residue, dissolved in 100 ml of water, was applied to a column (57 cm × 3.2 cm) of CM52, which had been converted to the H⁺ form by pretreating 400 g of it with 2 × 500 ml of 0.5 M-HCl and washing to a pH of 5.2. The loaded column was washed with 2.5 litres of water and the amine fraction was eluted with 2 litres of 0.5 M-HCl, all at 5°C.

After evaporating four times to remove HCl, the residue was dissolved in 6 ml of water. Aliquots (1 ml) were mixed with excess solid Na₂CO₃ and 1 ml of dansyl chloride (2 mg/ml in acetone). This time the reaction mixtures tended to separate into two layers so were vortex-mixed (for 2 min) seven times at 1 h intervals and then left for 16 h. After standing with aqueous proline (2 mg in 0.1 ml) for 3 h, the dansylated amines were extracted and chromatographed in two dimensions as usual except that the layers were 0.5 mm thick.

The relevant area of silica gel was extracted with 4 × 5 ml of ethyl acetate over 20 h; the product was taken to dryness and redissolved in 100 µl of ethyl acetate (Aristar grade). The six samples so obtained were recombined and chromatographed as a 16 cm wide streak on a 0.25 mm thick layer of silica gel 60H in solvent 4 [chloroform/triethylamine (5:1, v/v)]. The solvents used were the purest available (Spectrosol grade chloroform and AnalR grade triethylamine) and comparable care was taken in cleaning the glass plates used as layer support. After chromatography, the monodansyl-3-methoxytyramine zone was extracted for 18 h with 5 ml of acetone (Spectrosol grade)/water (5:1, v/v). This sample was evaporated, taken up in 0.1 ml of acetone (Spectrosol grade) and evaporated four times to remove excess water. It was finally dried over P₂O₅ at 1.3 × 10⁻³ Pa (10 torr) for 7 days before examination by n.m.r., giving a yield of 0.43 mg.

Synthesis of authentic mono- and bis-dansyl-3-methoxytyramine

Standard 3-methoxytyramine (10 mg) in 1 ml of water was mixed with 2 mg of Na₂CO₃, 16 or 32 mg of dansyl chloride and 1 ml of acetone. After 18 h, 7 or 14 mg of proline was added to the reaction mixture, the larger quantities of dansyl chloride yielding the bis-dansyl derivative. The required products were extracted with 2 × 5 ml of ethyl acetate 2 h later and taken to dryness in a stream of air at 50°C. They were finally purified by t.l.c. in solvent 4 as described in the previous section, except that the 60H layers were 0.5 mm thick.

[^1℃]Tyrosine feeding experiments

Untransformed N. tabacum tumours, as well as...
those transformed by A. tumefaciens strains B0542, ACH5, T37 and 181 (0.5g fresh wt. fragments of each), were incubated separately with 5μCi of [U-14C]tyrosine in 0.1 ml of water for 18 h at 20–25°C. Each tissue was ground up with 10ml of 70% (v/v) methanol and the amines were isolated, derivatized and chromatographed in two dimensions as for the determination of 3-methoxytyramine. However, CM52 was used in its H+ form and Merck pre-coated t.l.c. plates replaced the usual, less robust, self-adhesive layers. Radioautograms were prepared by exposing Kodak X-ray film to each plate for 2 months.

Results

The amines of crown galls and related tissues

Two-dimensional t.l.c. of dansylated amine fractions of N. tabacum crown gall tumours in solvents 1 and 2 yielded an average of 24 components, many of which co-chromatographed with dansyl derivatives of well known aliphatic mono-, bis- and polyamines. Mono- and bis-dansyl-p-tyramine and β-phenylethylamine were also present, together with ten 'unknowns'. The corresponding callus control appeared to lack three of the major 'unknowns'. One of the latter has now been identified as monodansyl-3-methoxytyramine, while another, designated T, is also a phenylethylamine derivative since, though it is chromatographically separable from bis-dansyl-p-tyramine, it has a very similar n.m.r. spectrum.

Chromatographic properties of mono- and bis-dansyl-3-methoxytyramine

Three solvents are necessary for the complete purification of these derivatives from crown gall preparations because two-dimensional chromatography does not resolve them adequately from dansylated forms of ammonia, putrescine and p-tyramine. The RF values of monodansyl-3-methoxytyramine in solvents 1, 2, 3 and 4 are 0.45, 0.34, 0.38 and 0.59 respectively, while the corresponding values for the bisdansyl derivative are 0.54, 0.39, 0.51 and 0.68.

Characterization of the amine isolated from N. tabacum/ACH5 tumours

The isolate co-chromatographed with authentic monodansyl-3-methoxytyramine on t.l.c. plates in all the solvents (1–4) tested. It gave a clear n.m.r. spectrum in (6H)chloroform at 100 MHz which was interpreted by comparison with spectra for authentic dansyl chloride, dansyl-methylamine, monodansyl-p-tyramine and p-tyramine, all obtained with a Jeol FSX-100 instrument.

Quoting chemical shifts (δ p.p.m.) relative to tetramethylsilane, the dansyl group of the isolate was represented by four multiplets in the range 7.0–8.6 (6H, naphthalene ring protons) and a singlet at 2.9 [6H, N(CH3)2]. The rest of the molecule appeared as 2.58 (2H, t, J 12Hz, -CH2-CH2-NH-), 3.10 (2H, q, J 20Hz, -CH2-CH3-NH-), 3.70 (3H, s, OCH3), 4.48 (H, t, J 12Hz, -CH2-CH3-NH-), 6.36 (2H, m, J 8Hz, phenolic ring protons) and 6.65 (H, m, J 8Hz, third phenolic ring proton). The 4.48 p.p.m. resonance disappeared on adding 3H2O, confirming its designation. The spectrum was consistent with the structure proposed and was identical with that given by synthetic monodansyl-3-methoxytyramine.

On a VG Micromass ZAB 1F instrument, the isolate gave a mass spectrum with major fragments at m/z 250 (α-fission), 263 (β-fission) (Reisch et al., 1968), 171 (from dansyl group) and a molecular ion at precise mass 400.1459 corresponding to an empirical formula of C21H24N2O4S (theoretical mass 400.1457). 3-Methoxytyramine levels in crown galls and related tissues

These are shown in Table 1. There is little doubt that 3-methoxytyramine was really present where indicated because all these extracts yielded fluorescent spots co-chromatographing with both mono- and bis-dansyl-3-methoxytyramine in solvents 1, 2 and 3. Moreover, the ‘dansyl-3-methoxytyramine’ obtained from N. tabacum/B0542 had an identical mass spectrum to the N. tabacum/ACH5 isolate. 3-Methoxytyramine dansylated equally efficiently in all extracts because about 90% of the monodansyl derivative was always formed. In the differentiated control samples the other amines were derivatized satisfactorily; doubling the proportion of dansyl chloride still gave no indication that any 3-methoxytyramine was present.

All the undifferentiated tissues were microbiologically sterile according to all the tests applied. The callus control cannot have been accidentally transformed because its growth was hormone-dependent and much slower than that of any crown gall. Moreover, it contained no known amines and metabolized [14C]tyrosine to a completely different spectrum of amines than did any of the crown galls studied.

[14C]Amines formed from tyrosine

The callus control gave 12 major metabolites and transformation caused 14C to be diverted away from four of them. However, transformation increased the 14C in the most highly labelled component, which chromatographed in the bis-dansyl-p-tyramine/compound T position. Label
was also present in the monodansyl-3-methoxytyramine region of all chromatograms.

Discussion

Dansylated amines are easier to purify than free amines as they chromatograph cleanly and can be readily freed from salt by solvent extraction. They can be detected at very low concentrations and their mass spectra usually show good molecular ions. Little has been published about their n.m.r. spectra, though dansylated phenylethylamines give easily interpreted spectra. Indeed this appears to be the first time that n.m.r. has been used to characterize a dansylated amine from a cell extract.

Table 1 indicates that the concentration of 3-methoxytyramine in the crown galls tested was 50–340 μg/g dry wt. This corresponds to the lower part of the concentration range for opines (40 μg–70 mg/g dry wt.) (Scott et al., 1979). However, the amine is not an opine. Its occurrence in the N. tabacum callus control shows it is not determined by bacterial DNA and its universal presence in a series of crown galls which, taken together, produce all known families of opines, indicates that it is not strain-specific.

In the plant kingdom, 3-methoxytyramine has previously only been recorded in the Cactaceae (Agurell et al., 1971; Crosby & McLaughlin, 1973; Pardanani et al., 1978; Strombon & Bruhn, 1978; Pumwangara & McLaughlin, 1981), a family showing abnormal morphology. It now seems that other higher plant groups have a latent capacity to produce this compound.

It is probably significant that the more disorganized the tissue and the higher its growth rate, the higher the level of 3-methoxytyramine. Table 1 shows that the amine cannot be detected in normal, differentiated, tissue and is present at an intermediate concentration in the callus control which grows at about 20% of the rate of a typical crown gall. The two results for Linum usitatissimum suggest that the effect is not dependent on the host species.

What has been observed is probably part of a more extensive change in amine metabolism: transformation not only enhances the relative importance of two unidentified components in addition to 3-methoxytyramine, but also influences the way in which tyrosine is metabolized to, presumably, aromatic amines.

There is a parallel with our results in mammals in that patients suffering from neuroblastoma typically show increased excretion of 3-methoxytyramine (Studnitz et al., 1963), formed here as a metabolite of dopamine. Dopamine is not known to act as a universal chemical messenger in plants as it does in animals, but it does remove the sugar inhibition of flowering response in Lemma gibba (Oota, 1974) and, together with the other catecholamines, synergistically enhances Lactuca sativa hypocotyl elongation induced by gibberellic acid (Kamisaka, 1979). Certainly phenylethylamines are physiologically active substances: 3-methoxytyramine itself is a precursor of the hallucinogen, mescaline, in cacti (Lundstrom & Agurell, 1969). Thus the changes described here may not all be secondary ones and could conceal control mechanisms of general importance in plant growth and differentiation.

We thank the Agricultural and Food Research Council of Great Britain for financial support and are very grateful to Dr. D. Farrant for obtaining and interpreting the n.m.r. spectra.
References