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N^2-(1-Carboxyethyl)methionine

A 'pseudo-opine' in octopine-type crown-gall tumours

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A novel methionine-containing plasmid-determined compound, N^2-(1-carboxyethyl)methionine (NCEM) has been identified in crown-gall tumours induced by octopine-type strains of *Agrobacterium tumefaciens*. NCEM is probably synthesized by octopine synthase. Cell-free preparations from octopine-type strains of *A. tumefaciens* can degrade NCEM; however, the bacterium cannot transport the compound into the cell, although these strains can take up and degrade the octopine family of opines.

INTRODUCTION

The Gram-negative bacterium *Agrobacterium tumefaciens* is the causative agent of crown-gall disease in dicotyledonous plants (Smith & Townsend, 1907). Pathogenic strains harbour a large tumour-inducing (Ti) plasmid (Van Larebeke *et al.*, 1974; Watson *et al.*, 1975), part of which (the T-DNA) is transferred to, and stably maintained in, the plant nuclear genome (Chilton *et al.*, 1977; Willmitzer *et al.*, 1980). Crown-gall tumours usually synthesize gall-specific compounds known as opines (Tempe & Petit, 1983; Chang *et al.*, 1983; Chilton *et al.*, 1984), whose synthesis is directed by the T-DNA (Bomhoff *et al.*, 1976; Ellis *et al.*, 1984); Ti plasmids can be classified according to the type of opine produced in the gall (Montoya *et al.*, 1977). Opines can serve as N and/or C and energy sources for those strains of *A. tumefaciens* that carry the appropriate opine-type plasmid. Thus, in Nature, opines may offer a selective advantage to those strains able to catabolize them (Bomhoff *et al.*, 1976; Ellis & Murphy, 1981; Montoya *et al.*, 1977). Octopine-type tumours synthesize the N^2-(D-1-carboxyethyl) derivatives of arginine (octopine), ornithine (octopinic acid), lysine (lysopine) and histidine (histopine). These opines can serve as N, C and energy sources for all octopine strains of *A. tumefaciens* and are selectively taken up by an inducible permease system (Klapwijk *et al.*, 1977). Nopaline strains induce tumours containing the N^2-(1,3-dicarboxypropyl) derivatives of arginine (nopaline) and ornithine (nopalinic acid). These strains can also utilize the octopine family of opines, provided they have been induced with nopaline (Klapwijk *et al.*, 1977). Octopine strains cannot, however, utilize the nopaline family of opines, even when induced with octopine. The bacterial catabolism of octopine or nopaline proceeds via arginine. We report here the occurrence of a novel methionine-containing compound whose synthesis in the crown-gall tumour is directed by octopine-type T-DNA.

EXPERIMENTAL

Initiation and culture of crown-gall tumours

Tumours were initiated on tobacco (*Nicotiana tabacum* cv. White Burley) seedlings and flax (*Linum usitatissimum*) seedlings with *Agrobacterium tumefaciens* strains A6, B6 (octopine-type), T37 and C58 (nopaline-type) as described by Butcher *et al.* (1980).

Chromatographic systems

One-dimensional descending paper chromatograms. These were run on Whatman 3MM paper in the following solvent systems: (I) propan-1-ol/35% (w/w) NH_3 (3:2, v/v); (II) propan-2-ol/90% (w/w) formic acid/water (20:1:5, by vol.).

Electrophoretograms. These were run on Whatman 3MM paper at a voltage gradient of 30 V/cm for 2 h in the following buffer systems: (III) 90% formic acid/acetic acid/water (1:3:16, by vol.), pH 1.89; (IV) 0.05 M-citric acid/sodium citrate, pH 5.0; (V) 0.05 M-sodium tetraborate, pH 9.5 (approx. 0.07 M-sodium tetraborate was adjusted to pH 9.5 with NaOH solution and diluted to 0.05 M). Sulphur-containing compounds were detected with the $PdCl_2$ reagent described by Toennies & Kolb (1951).

G.c.–m.s. N^2-(1-Carboxyethyl)methionine (NCEM) was methylated as described by Schlenk & Gellerman (1960). High-resolution g.c.–m.s. of the derivative was carried out by using a 12 metre OV-1 capilliary column. The carrier gas was He and the temperature program was 80–150 °C at 20 °C/min and 150–300 °C at 5 °C/min. The m.s. source temperature was 200 °C and the ionizing voltage was 70 eV.

Isolation of NCEM from tumour tissue

Tumour tissue was homogenized in water (10 ml/g fresh wt.), centrifuged, and the supernatant passed through a column of Duolite A101 resin (acetate form; 1 ml of resin/g fresh wt. of tissue extracted). The resin was washed with water, and anionic compounds, including NCEM, were eluted with 2 M-formic acid (10 bed volumes). The formic acid eluate was evaporated at 45 °C. NCEM was isolated from this extract by preparative paper chromatography in solvent system I (R_F 0.49).

Abbreviation used: NCEM, N^2-(1-carboxyethyl)methionine.

Isolation of [*methionine*-^{35}S]NCEM from tumour tissue

Tumour tissue was chopped into small pieces ($\sim$ 2 mm^3), blotted, and then flooded with [^{35}S]methionine (1028 Ci/mmol). After incubation at 20 °C for 12 h the tissue was ground with acid-washed sand, extracted with 10% (w/v) trichloroacetic acid (5 μl/mg fresh wt.) and centrifuged. [*methionine*-^{35}S]NCEM was isolated from the supernatant by preparative paper electrophoresis in system III ($R_{methionine}$ 0.32).

Cell-free synthesis of NCEM by tumour extracts

All operations were carried out at 4 °C unless stated otherwise. Tumour tissue was homogenized with 0.1 M-sodium phosphate buffer (0.1 M-Na_2HPO_4/0.1 M-NaH_2PO_4), pH 6.8, containing 2-mercaptoethanol (14 mM) and Polyclar (polyvinylpyrrolidone) (25 mg/ml) (2 ml of buffer/g fresh wt. of tissue). The homogenate was centrifuged (30000 *g*, 1 h) and the supernatant was concentrated by centrifugation (2000 *g*) through Amicon Centriflo ultrafiltration cones (CF 25) to a final volume of $\sim$ 1 ml. The concentrate was applied to a column (5 cm × 1 cm) of Sephadex G-25 (coarse grade) and was eluted with homogenizing buffer without Polyclar. Fractions equivalent to 1 bed volume were collected and analyzed for total protein (A_{280}). Those fractions containing protein were pooled and mixed with 9 vol. of incubation buffer (0.1 M-sodium phosphate, pH 6.8), containing NADPH or NADH (1.2 mM), pyruvic acid (15 mM) and L-methionine (87 mM). The mixture was incubated for 3 h at 25 °C. After incubation, 10% (w/v) trichloroacetic acid was added to a final concentration of 2% and the sample was centrifuged (8000 *g*, 10 min). NCEM was isolated from the supernatant by preparative electrophoresis (system III). When required, [^{35}S]-methionine was substituted for L-methionine in the incubation mixture.

Uptake of [*methionine*-^{35}S]NCEM, octopine and nopaline by *A. tumefaciens*

Bacteria were cultured overnight at 29 °C on either complete liquid medium (Vincent, 1970) or, if induction of the opine uptake and degradation systems was required, on minimal medium (Zevenhuizer, 1971) containing glucose (22 mM) and octopine or nopaline (0.7 mM). Strain C58$^-$ (no Ti plasmid) was cultured on complete liquid medium. Bacterial cells were harvested by centrifugation, washed twice in minimal medium and then resuspended in minimal medium containing glucose (22 mM) and octopine, nopaline (0.7 mM) or [*methionine*-^{35}S]NCEM. The final bacterial cell concentration was about 10^8 cells/ml. Bacterial cells were removed by centrifugation at time intervals up to 15 h, and samples of the supernatant were assayed as follows. Uptake of [*methionine*-^{35}S]NCEM was measured by liquid-scintillation counting and by paper chromatography (system I) followed by autoradiography. Uptake of octopine or nopaline was measured colorimetrically by using the α-naphthol/diacetyl reagent described by Johnson *et al.* (1974).

Degradation of [*methionine*-^{35}S]NCEM, octopine and nopaline by cell-free extracts from *A. tumefaciens*

Bacteria were grown for 15 h in minimal medium containing the relevant opine (0.7 mM) as the sole source of N, and glucose (22 mM). After harvesting by centrifugation, the bacterial pellet was resuspended in 0.5 ml of sonication buffer (0.1 M-sodium phosphate, pH 7.4, containing 0.1 mM-dithiothreitol) and sonicated for 2 min (30 s on/off mode). The sample was kept on ice during sonication. The resulting sonicated material was centrifuged (2000 *g*, 10 min, 4 °C) and incubations were carried out by mixing an equal volume of the supernatant with sonication buffer containing octopine, nopaline (4 mM) or [*methionine*-^{35}S]NCEM. Samples were incubated at 30 °C for 2 h and then analysed by h.p.l.c. after formation of derivatives with *o*-phthalaldehyde (Lindroth & Mopper, 1979), for the presence of arginine or [^{35}S]methionine.

RESULTS

The labelling patterns obtained after supplying [^{35}S]methionine to crown-gall tumours and untransformed callus (both derived from tobacco seedlings) show, after electrophoresis in system 3, the presence of a ^{35}S-labelled product only in the octopine-type tumour B6 (Fig. 1). Strain A6 gave the same result as B6, and C58 the same result as T37. In addition, tumours and callus derived from flax seedlings showed the same labelling patterns as those obtained from tobacco-derived tumours or callus.

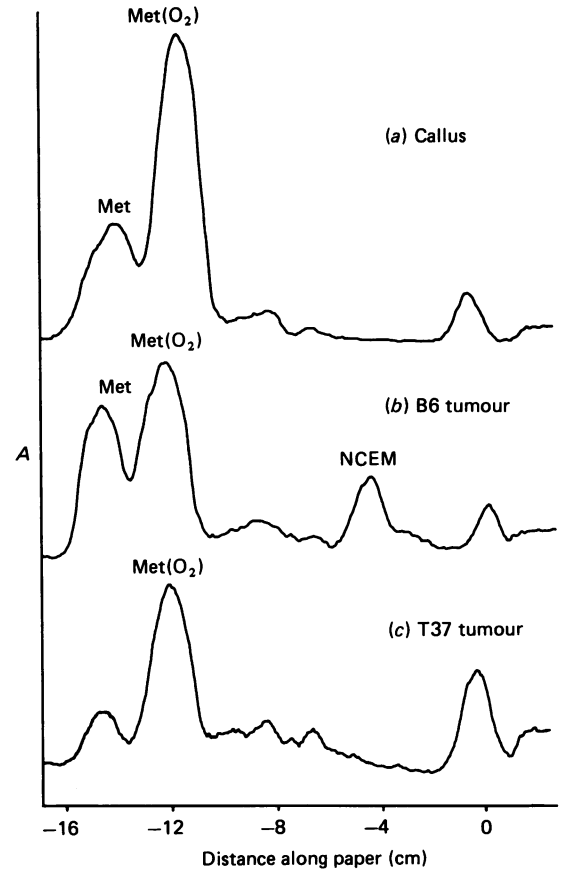
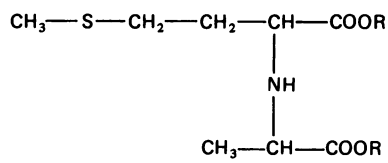


Fig. 1. ^{35}S-labelled metabolites present in the acidic fraction isolated from untransformed tobacco callus and crown-gall tumours induced on tobacco by *A. tumefaciens* strains B6 and T37 after incubation with [^{35}S]methionine for 12 hours

Metabolites were separated by paper electrophoresis and the autoradiogram obtained was scanned by a densitometer. Further abbreviation used: Met(O_2), methionine sulphone.

Paper chromatography of unlabelled tumour extracts showed the presence of a $PdCl_2$-positive spot present only in octopine-type tumours. This spot was chromatographically identical (systems I–V) with the ^{35}S-labelled product. The compound was isolated from the anionic fraction obtained from octopine-type-tumour extracts by preparative paper chromatography in system I (R_F 0.49), and was methylated. High-resolution g.c.–m.s. of this derivative yielded an accurate molecular mass of 249.1045, corresponding to an atomic composition of $C_{10}H_{19}N_1O_4S_1$. The fragmentation pattern obtained was consistent with the proposed structure:



R = H: N^2-(1-carboxyethyl)methionine (NCEM);
R = CH_3: methyl ester of NCEM

NCEM occurs in tumour tissue at approximately the same concentration as octopine (~ 100 μg/g dry wt.). Cell-free extracts from both octopine-type tumours (A6, B6) investigated synthesized a product from L-methionine and pyruvic acid that was chromatographically identical (systems I–V) with the naturally occurring product. The reaction was dependent on the presence of NADPH or NADH, methionine and pyruvate. No activity was found in the other tumour lines or in untransformed callus.

[*methionine*-^{35}S]NCEM, extracted from ^{35}S-labelled tumour tissue or synthesized by cell-free preparations from octopine-type tumours, was not taken up by any of the bacterial strains investigated, even though these strains could taken up and degrade their respective opine types (Table 1). Paper electrophoresis (system III)/autoradiography of the bacterial medium showed that all the radioactivity present in the medium during the experiment was associated with NCEM. Thus [*methionine*-^{35}S]NCEM was not taken up by the bacterium and subsequently exported from the bacterial cell in a different form.

Table 1. Uptake of octopine, nopaline and NCEM by uninduced (U) and induced (I) cultures of *A. tumefaciens*

Bacteria were pre-grown on either complete liquid medium (uninduced) or minimal medium containing octopine (A6, B6) or nopaline (T37, C58) (induced) before the uptake experiment. '+' indicated that more than 90% of the compound was taken up within the 15 h period; '−' indicates that less than 10% was taken up. Full details are given in the Experimental section. Abbreviations used: Oct, octopine; Nop, nopaline.

Bacterial strain	Plasmid type	Uptake of: Oct U	Oct I	Nop U	Nop I	NCEM U	NCEM I
A6, B6	Oct	+	+	−	−	−	−
T37, C58	Nop	−	+	+	+	−	−

Table 2. Degradation of octopine, nopaline and NCEM by cell-free extracts of *A. tumefaciens*

Opine catabolism was induced by culturing the bacteria in minimal medium containing glucose and either octopine (A6, B6) or nopaline (T37, C58). Strain C58$^-$, which cannot catabolize either opine, was cultured in complete medium. '+' indicates that more than 90% of the substrate was degraded within 2 h; '−' indicates that less than 10% of the substrate was degraded within this time. Abbreviations are defined in Table 1.

Bacterial strain	Plasmid type	Degradation of: Oct	Nop	NCEM
A6, B6	Oct	+	−	+
T37, C58	Nop	+	+	+
C58$^-$	None	−	−	−

In contrast, cell-free extracts from all octopine-type strains of *A. tumefaciens* were able to degrade both labelled and unlabelled NCEM when pre-grown with octopine (Table 2). The nopaline strains C58 and T37 could also degrade NCEM, but only after induction of nopaline oxidase by pre-growth with nopaline. Strain C58$^-$, which lacks plasmid pTiC58, could not degrade NCEM, octopine or nopaline. The major degradation product of NCEM was methionine.

DISCUSSION

NCEM has only been detected in octopine-type tumours, suggesting that this compound may be an opine. Its presence in octopine-type tumours induced on both flax and tobacco plants indicates that it is not a plant-species-specific product. The synthesis of NCEM by cell-free preparations from octopine-type but not from nopaline-type tumours, and the dependence of this reaction on the presence of pyruvate, methionine and NADPH/NADH indicates that NCEM is probably synthesized by octopine synthase. Otten (1979) has shown that lysopine dehydrogenase (octopine synthase) purified from octopine-type crown-gall tumours will accept methionine as a substrate. Thus the substrate range of this enzyme *in vivo* may now be extended to include not only the four basic amino acids (arginine, ornithine, lysine and histidine) present in the octopine family of opines, but also the neutral amino acid methionine.

However, NCEM cannot be regarded as a true opine, since it is not taken up by any *A. tumefaciens* strains tested, even after induction of the octopine or nopaline permease and oxidase systems. The block appears to exist at the permease level, since both octopine and nopaline oxidase can degrade NCEM *in vitro* at a similar rate to that obtained with octopine or nopaline. The major identifiable product of NCEM degradation is methionine. Thus both octopine and nopaline permease have a more restricted substrate range than the corresponding oxidase enzymes. The inability of any bacterial strain investigated to take up NCEM would indicate that the opine permease

system probably recognizes the basic amino acid moiety of the opine molecule rather than the side chain derived from the oxo acid.

We suggest that the term 'pseudo-opine' should be used to describe plasmid-encoded metabolites (such as NCEM) that cannot be utilized by the bacterial strain carrying that plasmid.

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