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# Agrocinopine A, a phosphorylated opine is secreted from crown gall cells

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**We showed that phosphorus-containing metabolites of crown gall tissues were all taken up by appropriate pTi$^+$ agrobacteria. All but one were also taken up by pTi$^-$ bacteria. This one compound, produced by nopaline-, but not by octopine-type tumours, was the only phosphorylated organic compound actively secreted by healthy crown gall cells, and it appears to be agrocinopine A. Testing crown gall cell exudates may be a general procedure for the identification of opines by transformed plant cells.**

*Key words:* Agrobacterium/agrocin 84/exudate/phosphorus/plant tumour

## Introduction

Opine is the generic term for compounds produced by plant cells which have been transformed by the insertion of T-DNA sequences from Ti or Ri plasmids of agrobacteria (Petit *et al.*, 1970; Kemp, 1976; Kemp *et al.*, 1978; Tepfer and Tempé, 1981; Chang *et al.*, 1983; Chilton *et al.*, 1984; Petit and Tempé, 1984). They serve as nutrients for agrobacteria growing in association with the crown gall tumerosphere (Tempé *et al.*, 1979,1984; Holsters *et al.*, 1982; Tempé and Petit, 1983). Their catabolism requires enzymes determined by the Ti plasmid, outside the T-region (Kemp, 1982; Petit *et al.*, 1983). Thus, an opine is a compound which satisfies three criteria. It is: (i) synthesised by transformed, but not by normal, plant cells; (ii) made available to the colonizing agrobacteria. Mechanisms of transfer are still undefined but genetic analysis of this process has been initiated (Messens *et al.*, 1985); (iii) metabolized by the inciting bacteria. In the best studied cases the catabolic genes have been shown to be carried by Ti plasmids homologous with the plasmid responsible for the transformation. Hence pTi$^-$ bacteria are unable to catabolize opines.

At the time this work was begun the only known opines were agropine, nopaline and octopine (plus homologues) (Firmin and Fenwick, 1978; Schell *et al.*, 1979; Petit *et al.*, 1978) but there were hints that other compounds might play such a role in the biology of some agrobacteria. Thus, there were strains whose plasmids induced tumours which produced no known opines and the existence of such tumours suggested either that production of opines was not an important aspect of tumerogenicity or that further opines remained to be identified.

A particularly intriguing observation was that carriage of nopaline Ti plasmids rendered their bacterial hosts sensitive to a phosphorylated nucleotide antibiotic, agrocin 84 (Kerr and Htay, 1974; Engler *et al.*, 1975; Tate *et al.*, 1979). These plasmids evidently determine an efficient and specific uptake mechanism for this compound (Murphy and Roberts, 1979). We attempted, by $^{32}PO_4^{3-}$ labelling of the metabolic products of crown gall cells, to identify the natural substrate of this permease. Among the phosphorylated compounds we observed, was one which we identified as agrocinopine A (agA) an enhancer of agrocin 84 activity described by Ellis and Murphy (1981) and shown to have the properties of an opine.

We demonstrate here that this compound is unique in being the only phosphorus-containing organic species actively secreted by tumour cells. It is taken up only by bacteria sensitive to agrocin 84 and produced only by gall cells transformed by such bacteria. AgA could supply carbon and phosphorus to the bacteria of the tumerosphere whereas octopine, nopaline and agropine supply carbon and nitrogen. All of these compounds must be made available to the tumerosphere bacteria. However it has been shown that retention of phosphorus by plant cells is extremely efficient and the escape of phosphorylated compounds occurs at almost immeasurably low rates (Bieleski, 1976; Bieleski and Ferguson, 1983). Thus, the efficient excretion of agA is a most exceptional process and implies an active and highly specific secretory mechanism in the transformed cells.

Since plant cells, normal or transformed, produce very many chemical compounds it is impractical to examine each separately to determine whether it is produced only by transformed tissues. By limiting our study to chemical species which are secreted we can greatly simplify our task. Thus we propose, as a protocol for the identification of opines, to grow bacteria-free transformed tissue as callus in tissue culture medium, to separate the exhausted medium, with as little damage to the plant cells as possible, to fractionate the organic chemicals secreted by the cells and to check the ability of pTi$^-$ bacteria to remove these compounds from the medium. Any compound not efficiently removed by such bacteria is a candidate for further testing. This approach involves the assumption that a cell growing as a callus in tissue culture medium is physiologically analogous to one growing as part of a crown gall. This assumption is probably an oversimplification but should be valid as a first approximation.

Although we have limited our study to phosphorus-containing opines, it is likely that testing exudates by using appropriate radioactive labels (e.g., $^{14}C$, $^{3}H$ or $^{35}S$) the whole range of organic compounds could be screened and all opines identified. The use of a variant of our technique for assaying agropine and mannopine, (using a chemical stain rather than a radioisotope) has already been exploited by Salomon *et al.* (1984).

## Results

*The $^{32}P$-labelling of metabolic products of crown gall tissue*

*Principles of the analytical technique.* Transformed cells were grown as callus floating in liquid medium into which $^{32}PO_4^{3-}$ can be incorporated. After appropriate growth intervals the liquid can be removed without handling the plant tissue. Thus, no cell damage can occur and clear distinction of intra- and extra-cellular material can be made. Intact plant cells synthesise arginine but retain this amino acid intracellularly. Thus, as a control of cellular integrity the exhausted medium was also subject to electrophoresis

and arginine was monitored. Tumour cells convert arginine to octopine or nopaline, and the opine is efficiently secreted into the extracellular medium (Messens *et al.*, 1985).

*Secretion of a single phosphorylated compound.* The secretion of phosphorylated compounds was followed by analysing the growth medium of the cell lines listed in Table I. When the undamaged calluses were floating in liquid MS medium, the nopaline tumour lines W38C58 and W38T37 displayed an efficient secretion of one single $^{32}$P-product as shown in Figure 1A, lane 2, of the thin layer electrophoresis. This product was subsequently identified as agA. No $^{32}$P-containing compounds were secreted by any of the other cell lines of Table I (data not shown). In Figure 1A, lane 1, the cell extract from washed tumour cells is shown, as a control. These results show an efficient uptake of [$^{32}$P]phosphate after less than 1 week incubation (Figure 1A). Most of the compound synthesised during the period of labelling was secreted into the extracellular medium.

**Table I.** Wild-type tumour lines derived from *Nicotiana tabacum* cv. Wisconsin 38

| Specifications | | Reference |
|---|---|---|
| W38C58 | Uncloned tissue induced by strain C58 | Maliga *et al.* (1973) |
| W38B6S3 | Uncloned tissue induced by strain B6S3 | Leemans *et al.* (1982) |
| W38T37 | Clone from a teratoma line initiated by strain T37 on tobacco | Braun and Wood (1976) |
| W38A66 | Uncloned tissue induced by strain A66 | Binns *et al.* (1982) |
| W38 | Tobacco callus from untransformed plant tissue | Lemmers *et al.* (1980) |

*All but one of the phosphorylated products of crown gall cells are taken up by pTi$^-$ agrobacteria.* As can be seen from Figure 1B the $^{32}$P-labelled tumour cells produced numerous phosphorylated compounds. A total cell extract was submitted to preparative paper electrophoresis and eluted in zones (A, B. . . etc.). Figure 1B shows a second dimension electrophoretic separation of the W38C58 cell extract in a different buffer system. Each lane (A, B. . . . . K) corresponds to the elution zones with decreasing mobilities from the first dimension preparative electrophoresis.

Each of the tumour lines listed in Table I was labelled for 1 week. The fractions (A, B. . . . etc.) were supplied to various *Agrobacterium* strains. Opines were identified by monitoring the catabolism of each fraction by concentrated, logarithmically growing isogenic pTi$^+$ and pTi$^-$ strains of *A. tumefaciens*. With a single exception, all $^{32}$P-containing metabolites were removed from the media by all bacteria, after an incubation period of 16 h. The exceptional compound, located at the upper part of the heavy double spot in lane E of Figure 1B, indicated with a vertical bar, was shown to correspond with the secreted compound

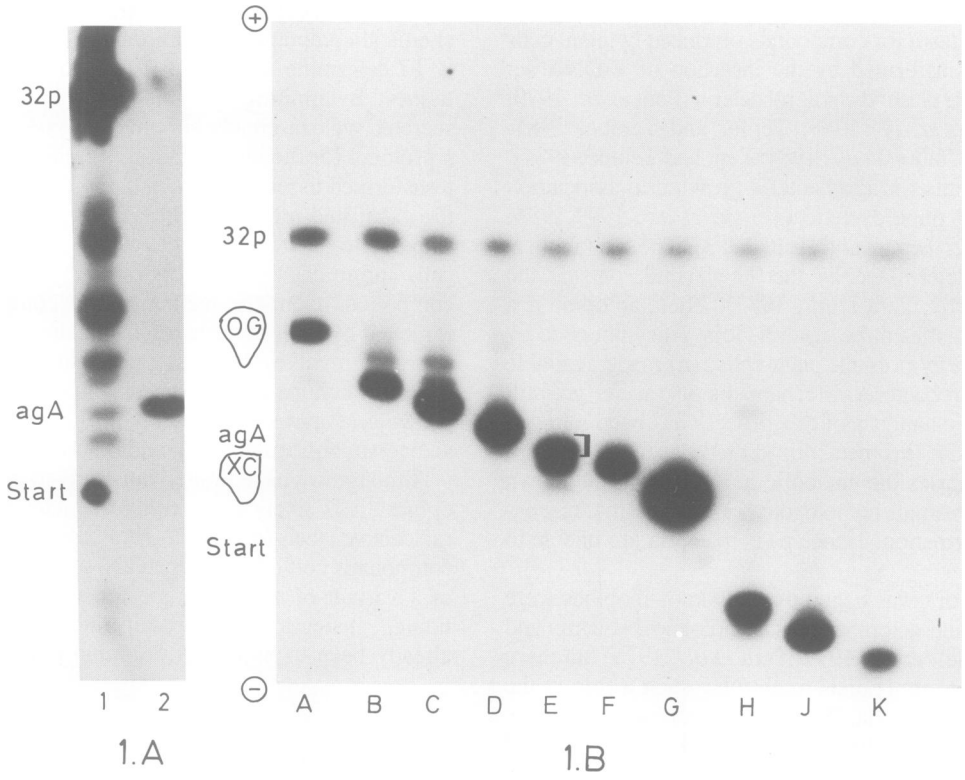


**Fig. 1. (A)** Agrocinopine (agA) is efficiently secreted into the growth medium. Samples of W38C58 tumour tissue (1 mm$^3$ in 25 $\mu$l MS medium, 2.5 $\mu$Ci $^{32}$P, pH 5.8), were analysed by thin layer electrophoretic separation, pH 5.0, 0.05 M citrate, 1 h, 400 V, as described in last section of the Results. **Lane 1,** Cell content after a $^{32}$P labelling period of 1 week. The thoroughly washed cells were opened by crushing in phenol and 2 $\mu$l of the aqueous phase spotted. **Lane 2,** 2 $\mu$l of exhausted growth medium, were spotted after the same labelling period. Cells were removed by low speed (500 r.p.m.) centrifugation; agA at (+0.32) and [$^{32}$P]orthophosphate (+1.00). **(B)** Preparative electrophoretic purification of soluble cell metabolites. 1 g W38C58 tumour tissue in 1.5 ml MS medium was labelled with 1.5 mCi [$^{32}$P]phosphate pH 5.8 for 1 week. Cells and medium were crushed in phenol and the aqueous phase spotted (300 $\mu$l/sheet Whatman 3MM). The elution zones of the pH 6.5 separation were numbered (A, B, C. . . . K) in a decreasing mobility order. AgA, shown with a vertical bar is found in the upper zone of the heavy spot in **lane E** (+0.44) in the subsequent separation on Whatman 3MM paper at pH 5.0, 0.05 M citrate, 1 h, 1.500 V; reference markers: XC, xylene cyanol (+0.34); OG, orange G (+0.70); $^{32}$P, [$^{32}$P]orthophosphate (+1.00).

(agA). It was synthesized by both nopaline-synthesizing lines but not by the octopine-synthesizing tumour lines. It was metabolized by agrobacteria carrying nopaline Ti plasmids, but not by bacteria lacking Ti plasmids nor by those with octopine plasmids. It was found as a single $^{32}P$-spot in the exhausted medium, with only traces of free phosphate label if cells were undamaged.

*The secreted compound is agrocinopine A*

*Chemical indications.* To characterise this compound we needed an efficient preparative procedure. Since the compound is so efficiently secreted into the extracellular medium the supernatant of a fully grown cell suspension culture was chosen as a source for its isolation. The yield was 10 – 15 times higher than for cell extraction. No breakdown product (as monitored by $^{32}P$-labelling) contaminated the preparation if the filtrate was phenolized immediately before its concentration and gel filtration on a P2 column. The gel filtration pattern (Figure 2) shows that the compound elutes as a molecule with a mass >1800 daltons. It was possible to isolate 20 mg crude and finally 8 mg pure product out of a 3 litre supernatant of W38C58 cell suspension culture. The complete isolation procedure together with a proposed structure will be published elsewhere. This compound proved to be agA by the following tests (Ellis and Murphy, 1981): (i) both are produced only by cells transformed by nopaline metabolizing agrobacteria; (ii) both are phosphorylated sugar derivatives; (iii) the *acc* gene is needed for the uptake of both compounds; (iv) pre-incubation of *acc*$^+$ agrobacteria with either compound increased susceptibility to agrocin 84. This is due to the induction of a permease (Murphy *et al.*, 1981); (v) colour tests for hexoses and pentoses on our compound and a sample of purified agA (generously provided by Dr. Ellis) gave identical results. Identity was established on the criteria of co-migration during electrophoresis in six different buffer systems and three chromatography systems (Table II and Discussion). This is illustrated in Figure 3 where a $^{32}P$-autoradiogram and a colour test as used by Ellis and Murphy (1981) shows the overlapping of the spots.

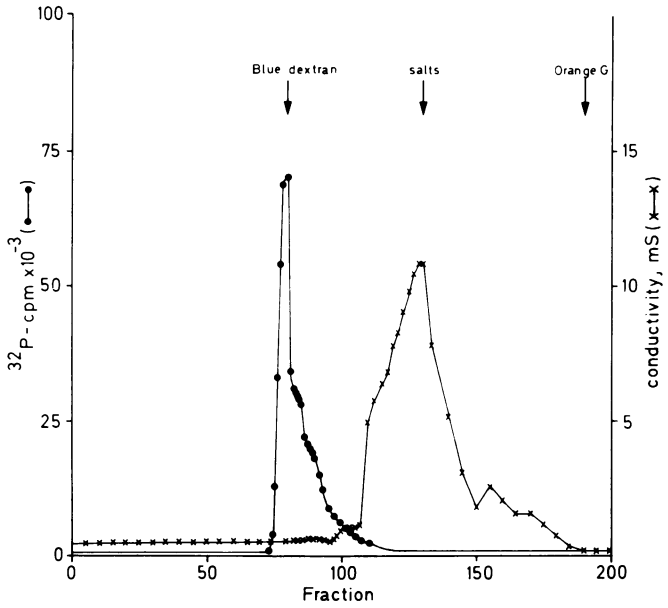


**Fig. 2.** Agrocinopine A is a compound with a mol. wt. >1800 daltons. A concentrated aliquot (50 ml) from 3 litres of supernatant from W38C58 cells labelled with purified [$^{32}P$]agrocinopine 0.8 x 10$^6$ c.p.m. and colour marker (5 mg each) was gel filtered onto a 5 x 100 cm column of Bio-Gel P2. The column was equilibrated and eluted with 0.01 M triethylammonium bicarbonate, and fractions of 10 ml were collected.

We, therefore, conclude that our opine is identical with agA.

*Genetic determination of the synthesis and secretion of agrocinopine A.* A series of tumour lines carrying derivatives of the T-DNA sequence of pTiC58 with deletion or insertion mutations at well defined sites have been described (Holsters *et al.*, 1980; Joos *et al.*, 1983; Zambryski *et al.*, 1983). Twenty one of these mutations were studied and three (pGV3315, pGV3170 and pGV3850) abolished the synthesis of agA whereas the others did not. This allows us to conclude that the gene (or genes) for agA lies in the right part of the *Hind*III-14b fragment. A single RNA transcript of this region has been identified (Willmitzer *et al.*, 1983), and this gene has been designated *acs* (*a*grocinopine *s*ynthase) (Joos *et al.*, 1983).

Experiments with tumour tissue supplied with radioactive phosphate showed that agA was synthesized and subsequently secreted efficiently into the extracellular medium. All mutant tumour lines which produced agA, secreted this compound efficiently. We are thus unable to assign the agA secretion function to any T-DNA gene.

**Table II.** Co-migration of agrocinopine A and the phosphorylated opine isolated by our technique

| | Relative mobilities ($^{32}PO_4$=1.00) Electrophoresis[a] | | | | | | $R_f$ values Chromatography[a] | | |
|---|---|---|---|---|---|---|---|---|---|
| | pH 1.8 | pH 3.5 | pH 5.0 | pH 6.5 | pH 9.0 | pH 9.2 | A | B | C |
| Whatman 3MM | | | | | | | | | |
| Agrocinopine A | +0.75 | +0.44 | +0.44 | +0.45 | −0.15 | +0.38 | 0.27 | 0.36 | 0.39 |
| Arginine | −7.75 | −0.72 | −0.36 | −0.53 | −0.91 | −0.22 | 0.32 | 0.40 | 0.25 |
| $^{32}PO_4^{3-}$ | +1.00 | +1.00 | +1.00 | +1.00 | +1.00 | +1.00 | 0.44 | 0.11 | 0.00 |
| Orange G | +4.65 | +0.83 | +0.70 | +0.77 | +0.65 | +0.67 | 0.74 | 0.78 | 0.78 |
| Xylene cyanol | +0.30 | +0.39 | +0.34 | +0.37 | 0.00 | +0.28 | 0.81 | 0.90 | 0.91 |
| Our opine (agA) | +0.75 | +0.44 | +0.44 | +0.45 | −0.15 | +0.38 | 0.27 | 0.36 | 0.39 |
| Cell MN300 | | | | | | | | | |
| Agrocinopine A | +0.21 | +0.57 | +0.32 | +0.32 | 0.00 | +3.50 | 0.31 | 0.40 | 0.45 |
| Arginine | −1.75 | −1.05 | −0.60 | −1.10 | −2.47 | −4.50 | 0.33 | 0.36 | 0.08 |
| $^{32}PO_4^{3-}$ | +1.00 | +1.00 | +1.00 | +1.00 | +1.00 | +1.00 | 0.42 | 0.00 | 0.00 |
| Orange G | +2.00 | +0.85 | +0.91 | +0.76 | +1.73 | +9.25 | 0.81 | 0.64 | 0.64 |
| Xylene cyanol | +0.46 | +0.30 | +0.30 | +0.16 | 0.00 | +2.00 | 0.89 | 0.77 | 0.70 |
| Our opine (agA) | +0.21 | +0.57 | +0.32 | +0.32 | 0.00 | +3.50 | 0.31 | 0.40 | 0.45 |

[a]As described in Materials and methods.

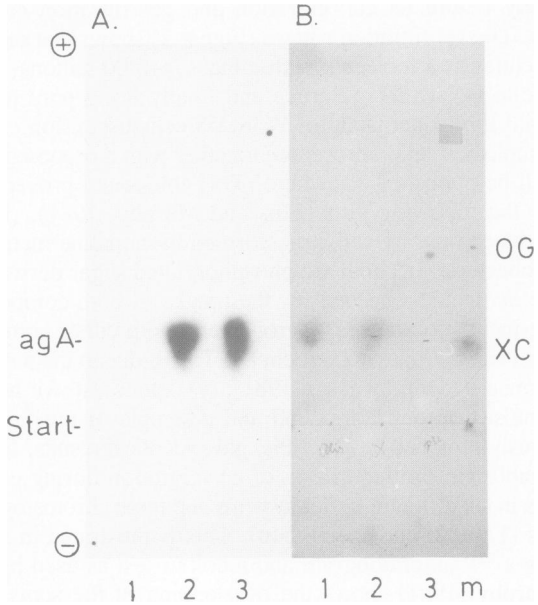


**Fig. 3.** Identity of agA on the criteria of co-migration: one example. (**A** and **B**) electrophoresis at pH 3.5 in 5% acetic acid, 0.5% pyridine, 1 h on Whatman 3MM paper. **Lane 1** is purified agA kindly supplied by Dr. J. Ellis. **Lane 2** is a mixture of this agA preparation and our material. **Lane 3** contains our isolate alone. In **Lane m** are the colour markers, xylene cyanol, XC (+0.39) and orange G, OG (+0.83). **Part A,** autoradiogram. **Part B,** the paper strip has been sprayed with *p*-anisiline HCl (Hough *et al.*, 1950). The centre of a brown spot (+0.44) coincides completely with the centre of the darkening in **lane 2 part A**.

*Catabolism of agrocinopine A.* Four mutants of the Ti plasmid of *A. tumefaciens* C58 isolated as conferring resistance to agrocin 84 (*ags*$^R$) (Holsters *et al.*, 1980), two transposon insertions (pGV3103 and pGV3108) and two deletion mutants (pGV3119 and pGV3120) were found to be unable to catabolize agA. These insertions define an *a*grocinopine *c*atabolism (*acc*) region at ~87 Md, as the genetic map of pTiC58 is usually drawn (Holsters *et al.*, 1980) and genetic analysis of *ags*$^R$ and *acc* mutants is in progress. Mutants deficient in agrocinopine catabolism isolated by M. Sorman also confer resistance to agrocin 84. Other mutations isolated as resistant to agrocin 84 were found to be *acc*$^-$ (M. Sorman, unpublished results). These results suggest that a single mechanism transports both agrocin 84 and agrocinopine into the cell confirming the conclusions of Ellis and Murphy (1981) and Murphy *et al.* (1981).

*Identification of agrocinopine A from transformed cells*

*Kinetics of $^{32}$P-labelling and secretion.* Determination of the optimal labelling time for detection of [$^{32}$P]agrocinopine is illustrated by Figure 4. The turnover of the [$^{32}$P]orthophosphate and the appearance of agA was monitored over a period of 20 days. Each sampling contained the $^{32}$P-compounds of the tumour cells and the extracellular medium assayed together. Samples were taken from the water layer of the homogenized and phenolized slurry of cells and medium.

The difference between the kinetics of agA synthesis and those of octopine or nopaline synthesis are striking. The latter compounds are synthesised from labelled arginine within 24 h whilst several days incubation are required for the labelling of agA (Figure 4). Several compounds are labelled with $^{32}$P more rapidly than agA and these may be precursors. This suggests that the synthesis of agA may be a multistep process in contradiction to the one-step process proposed by Ryder *et al.* (1984). Nevertheless, a large proportion of the added $^{32}$P was eventually incorporated in agA (Figure 4). Because this is efficiently secreted, it constitutes a drain on the intracellular phosphate pool.

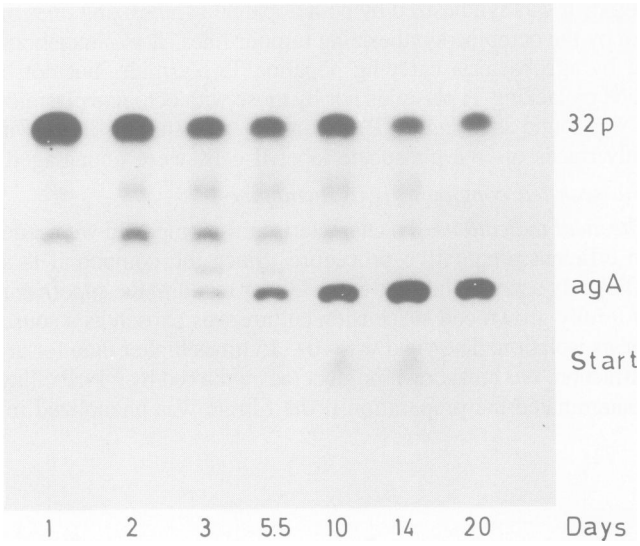


**Fig. 4.** Incorporation and turnover of the $^{32}$P-label as a function of time. At the time intervals indicated in the lanes, 2 μl samples were taken from the aqueous phase of the homogenized and phenolized slurry of batches of ~50 mg W38C58 cells grown in 100 μl MS medium supplemented with 1 μCi $^{32}$P at pH 5.8 and subjected to thin layer electrophoresis at pH 5.0, 0.05 M citrate, 1 h, 400 V; agrocinopine A (agA) at (+0.32) and [$^{32}$P]orthophosphate (+1.00).

$^{32}$P-Labelled agA was stable for at least 20 days in the presence of either normal or transformed plant tissue (results not shown).

*Optimized microprocedure for identification of agrocinopine A.* After agA was identified and its secretion demonstrated, we developed a routine micro-test which is reliable and at least 100 times more sensitive than any of the existing chemical stains for opines.

In all but one of the electrophoretic or chromatographic systems tested, agA co-migrated with one or more other $^{32}$P-labelled compounds from the total tumour extract. The exception was electrophoresis on thin layer cellulose plates in 0.05 M citrate buffer at pH 5.0. This is an optimal system and avoids errors from spots arising with the highly labelled (and sometimes damaged) cells. Thus, a clear lane, as in Figure 1A, implies undamaged healthily growing cells. We used the minimal amount of transformed cells that could be handled, usually ~1 mm$^3$ of tissue floating in the tip of our Eppendorf tube in 25 μl $^{32}$P-labelled (2.5 μCi) MS medium.

A labelling time of 5–7 days was optimal. After that time, 1 or 2 μl of extracellular medium were analysed by thin layer electrophoresis at pH 5.0. Three hour autoradiographic exposure usually showed the presence of agA unambiguously, but negative results were often confirmed by longer exposures. Preliminary phenol extraction removed unwanted substances from the samples. This treatment markedly improved the chromatographic separation.

We have established that octopine and nopaline are secreted efficiently whereas arginine can be taken up (monitored with [$^3$H]arginine) but is not secreted during the following 72 h. Damaged or necrotic cells, however, release arginine, so, one can detect cellular damage and routinely assay extracellular medium to confirm the absence of [$^3$H]arginine (Messens *et al.*, 1985).

## Discussion

We have shown that among the phosphorylated low molecular mass products from crown gall cells only one was actively secreted into the extracellular medium. This compound could not be removed from that medium by pTi$^-$ agrobacteria. These two properties, thus, proved extremely stringent criteria for an opine. This compound, once recognized as a possible opine, was shown to have all the properties to be expected of such a compound.

A gene has been located on the pTiC58 plasmid which determines a permease required for the uptake of agA or agrocin 84 (Murphy *et al.*, 1981). This gene is sometimes termed *acc* (*a*grocinopine *c*atabolism) and sometimes *ags* (*ag*rocin 84 *s*ensitivity). The arguments presented above indicate that the phosphorylated opine is identical with agA.

Ryder *et al.* (1984) have recently proposed a structure for agA; a phosphodiester of sucrose and L-arabinose with a molecular mass of ~553 daltons. Such a structure is not compatible with the mass determination presented in this paper. Perhaps their molecule is a derivative of the natural opine, possibly produced by the action of an enzyme activated during the isolation procedure. Ellis and Murphy (1981) reported that agA is susceptible to hydrolysis by mildly acid conditions or by the activity of pTi$^-$ agrobacteria, the product retaining biological activity.

By virtue of its labelling with $^{32}$P, synthesis of agA can be detected and assayed with great precision. Thus, it supplies an ideal marker of transformation and these advantages have been exploited in the recent demonstration of transformation of monocotyledons (Hernalsteens *et al.*, 1984). In the preliminary experiments we characterized all the soluble $^{32}$P-containing compounds in the callus tissue, and showed that the pTi$^-$ agrobacteria were able to use efficiently all the phosphorylated products of untransformed cells. However these compounds are retained by viable cells and so are not readily available to the bacteria. Thus, their metabolic role contrasts sharply with that of opines. Tumour cells secrete agA efficiently and we expected to be able to locate, within T-DNA of pTiC58, a gene (or genes) responsible for this secretion, as was the case with octopine and nopaline (Messens *et al.*, 1985). However, although a set of deletions covering the whole length of the T-DNA were tested, all the cells which synthesised agA also secreted the compound. This could be due to passive leakage (unlikely for so large a compound) or to secretion by a mechanism encoded by a gene (or genes) of the plant genome. If the secretion is determined by the T-DNA, two models seem possible; either two separate agrocinopine secretion mechanisms are encoded and both must be deleted to abolish the phenotype or the secretion could be a pleiotropic function of one of the genes (*roi, shi* or *acs*) essential for the establishment of an agA-synthesising tumour cell line. We do not have sufficient evidence to choose between these alternatives.

The very efficient conversion of inorganic phosphate to agA and the secretion from tumour cells suggest that, for a plant growing on a soil low in available phosphorus, agrocinopine-producing tumours could be specially damaging pathologies. It may, however, be an advantage to the associated bacteria that tumour cells should provide a phosphorus-containing nutrient as well as the nitrogenous nopaline. This distinguishes the nopaline tumours from octopine tumours where only nitrogenous compounds are provided.

According to our current understanding of the biology of *A. tumefaciens*, the production of opines by transformed plant tissue is a major nutritional resource for the bacterium and if we are to understand the ecology of this species we must learn the range of opines produced and how agrobacteria exploit them. Thus, a systematic approach to the identification of opines is required. However, the chemical structures of opines are too diverse for any attempt at direct identification and it is only by the application of biological criteria that we can hope for a systematic survey.

We consider that our techniques may prove useful in the study of opines produced by plant tissues. Several new opines have recently been described in tissues transformed by agrobacteria (Chang *et al.*, 1983; Chilton *et al.*, 1984; Petit and Tempé, 1984) and others may be still undiscovered. We also suggest that systematic surveys of plant hypertrophies induced by other bacteria may also involve analogous compounds which could be studied in a systematic survey that used our procedure. For example, rhizobia can produce tumour-like structures (MacGregor and Alexander, 1971), may be able to introduce DNA sequences into plant genomes (Broughton *et al.*, 1984), and induce the synthesis by plant cells of uncharacteristic compounds which can be metabolized by the same bacteria strains (Virtanen and Laine, 1939; Tempé and Petit, 1983). Other bacteria known to produce abnormal plant growths are *Pseudomonas savastanoi* (Comai and Kosuge, 1983) phyllobacteria (Fletcher and Rhodes-Roberts, 1979; Knösel, 1984) and corynebacteria (Murai *et al.*, 1980; Vidaver and Starr, 1981).

These phenomena may be fundamentally different from crown gall in requiring continuous co-existence of bacteria (or bacterioids) with the plant tumours, but the production of opine-like bacterial nutrients may be an essential feature of such interactions and it should prove possible to modify the techniques presented to seek such compounds in all bacterially-influenced plant tissues.

## Materials and methods

*Chromatography and paper electrophoresis*

Thin-layer chromatography was performed on Polygram Cell 300 plastic sheets (Macherey-Nagel and Co., Düren, FRG) with the solvent systems A (n-butanol; acetic acid; pyridine; water; 15:3:10:12 v/v/v/v), B (n-propanol; 25% $NH_4OH$, 3:2 v/v) or C (isopropanol; pyridine; water 2:2:3 v/v/v).

Spots were detected visually or under u.v. illumination, after spraying with a fresh staining mixture as described below for paper chromatography. Descending paper chromotagraphy was performed using Whatman 3MM paper in the same systems A, B or C, for 14 h. Spots were detected by dipping the dried chromatograms in equal amounts of 0.02% phenantrachinon in ethanol: and 10% potassium hydroxide in 70% ethanol, and visually detected under u.v. illumination (Yamada and Itano, 1966). Fluorescent spots already present before staining were marked with a pencil. Colour tests with phloroglucinol for pentoses and with L-cysteine-sulphuric acid for hexoses were as described by Dische (1962). The colour test with *p*-anisidine-HCl was as described by Hough *et al.* (1950).

Paper electrophoresis or thin-layer electrophoresis was carried out using Whatman 3MM paper at either pH 1.8 (2% formic acid, 8% acetic acid), pH 3.5 (5% acetic acid, 0.5% pyridine), pH 5.0 (0.05% M citric acid adjusted with caustic soda), pH 6.5 (0.5% acetic acid, 10% pyridine), pH 9.0 (0.1 M ammonium bicarbonate adjusted with $NH_4OH$), pH 9.2 (0.05 M boric acid adjusted with caustic soda), in a high-voltage apparatus using Varsol as heat exchanger, as described previously (Ryler *et al.*, 1955). [$^3$H]Arginine and [$^3$H]opines are detected by dipping the chromatogram in 5% 2,5-diphenyloxazole (PPO) in diethylether, followed by a 24 h or longer fluoro-autoradiography to Fuji RX safety film between intensifying screens at −80°C. [$^{32}$P]Phosphate and derivatives were detected by autoradiography at room temperature. Standards included Xylene cyanol (−1 ionic charge), Orange G (−2 ionic charge), phosphoric acid (−3 ionic charge), octopine (neutral), nopaline (+1 ionic charge), and arginine (+2 ionic charge). Migration distances quoted are for 45 min at 2000 V (40 V/cm) using Whatman 3MM paper and 45 min at 400 V (25 V/cm) using polygram Cell 300 plastic sheets.

*Bacterial strains*

*Agrobacterium* strains with mutant pTiC58 plasmids (Holsters *et al.*, 1980; Joos *et al.*, 1983; Zambryski *et al.*, 1983) or mutant pTiB6S3 plasmids (Leemans *et al.*, 1982) were grown in YEB medium to mid-log phase at 28°C. Subsequently, bacteria were concentrated by pelleting at 6000 r.p.m. for 10 min and resuspended in 0.1 volume of minimal A medium (Miller, 1972). A66 strain was from Binns *et al.* (1982) and T37 from Braun and Wood (1976).

*Crown galls*

Tissue cultures from the crown galls induced with the mutant strains mentioned above were obtained as described in the references of Table I. Tissue cultures were grown at 24°C in 16 h/day illumination at 2000 lux on top of agar in 10 cm Petri dishes with 30 ml Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), 0.6% of agar, supplemented with antibiotics: 0.5 mg/ml cefotaxime (Claforan$^R$, a kind gift of Hoechst, Belgium), or 1 mg/ml vancomycin (Sigma), or 1 mg/ml carbenicillin and 0.1 mg/ml rifampicin, some in combination with 50 units/ml nystatin as anti-fungal agent. Untransformed W38 callus tissue was grown on an MS medium supplemented with 2 mg/ml naphthalene acetic acid (NAA) and 0.1 mg/l kinetin (Lemmers *et al.*, 1980). Mutants in gene 4 (*roi*) were supplemented only with 0.1 mg/l kinetin. Mutants in gene 1 or 2 (*shi*) were supplemented with 2 mg/l NAA. Mutants affecting both 1 or 2 and 4 are treated as the untransformed W38 callus.

*Preparation of total cell extracts for the detection of [$^{32}$P]opines*

1 g of bacteria-free crown gall tissue culture grown on top of petri dishes was transferred to 1.5 ml MS medium, supplemented with 1.5 mCi $^{32}$P-carrier-free phosphate (IRE, Fleurus, Belgium). The radioactive phosphate batch was adjusted to pH 5.8 with 0.1 volume 1 M 4,morpholino-ethanesulfonic acid (MES) adjusted to pH 5.8 with sodium hydroxide. After incubation for 7 days cell extracts were made by adding 1 g pure phenol and crushing the tissues at 0°C. The aqueous phase, cleared by centrifugation, was fractionated by preparative electrophoresis at pH 6.5, 20 V/cm, for 90 min on Whatman 3MM paper. $^{32}$P-containing bands, located by autoradiography, were excised and eluted. The elution zones were numbered with capital letters in a decreasing mobility order.

The eluates were freeze-dried and redissolved in 100 µl 0.01 M MES buffer, pH 5.8. The metabolites analyzed for opine detection were shown in a subsequent separation pattern at pH 5.0, Figure 1B.

*Incubation of Agrobacterium strains with labelled tumour cell extracts*

Ti$^+$ and Ti$^-$ *Agrobacterium* strains were grown in YEB (5 ml) and concentrated in minimal A (0.5 ml) as described above, and then supplemented with a fraction (A, B, C,. . . ) of the $^{32}$P-labelled tumour extracts. The removal of the $^{32}$P-compounds was monitored by electrophoretic analysis at pH 5.0 at regular incubation intervals.

*Isolation of crude agA and estimation of molecular mass*

Twice electrophoretically purified [$^{32}$P]agrocinopine A (agA), showing up as a single radioactive band in all systems, was obtained from an electrophoretic purification at pH 5.0 from the freeze-dried eluate of the upper part of zone E from the preparative pH 6.5 electrophoresis of $^{32}$P-labelled W38C58 cells.

Sterile tobacco crown gall W38C58 cells were grown in liquid medium in 5 litre roller flasks containing 1 litre of culture. 3 litres supernatant were isolated by filtration through a gauze and loose cotton wool from fully grown cell suspensions; this liquid was immediately phenolized. The aqueous phase was extracted with diethyl ether, concentrated *in vacuo* to a final volume of 150 ml, and cleared by a 70% ethanol precipitation at −20°C. The procedures were repeated twice and the final aliquot (50 ml) was loaded on a 5 x 100 cm column Bio-Gel P2. The column was equilibrated and eluted with 0.01 M triethylammonium bicarbonate and fractions of 10 ml were collected. Monitoring was facilitated by adding twice electrophoretically purified [$^{32}$P]agA (0.8 x 10$^6$ c.p.m.) blue dextran (5 mg), and Orange G (5 mg). Fractions 74−97 contained the blue dextran and the radioactivity. These fractions were pooled and reloaded onto the same column. Finally the peak fractions were freezed-dried to yield ~25 mg crude product

*Incubations with agrocinopine A for the detection of agrocinopine-catabolizing minus (Acc$^-$) strains*

All mutant C58 *A. tumefaciens* strains (Holsters *et al.*, 1980) were grown and concentrated as described above, and subsequently supplemented with twice electrophoretically purified [$^{32}$P]agA. After incubation for 16 h at 28°C the supernatant of the bacterial cultures were analyzed by electrophoresis at pH 5.0.

*Micro-method for detection of agA*

After 5 days incubation in a $^{32}$P-containing MS medium and transfer of the undamaged $^{32}$P-labelled tumour tissue to fresh MS medium the secretion of $^{32}$P-labelled agA was monitored. Samples of the surrounding medium were taken at regular intervals. Tissues were lifted with care from the original incubation mixture, using a plastic needle and remaining cells and cell debris were discarded by low speed (500 r.p.m.) centrifugation and the radioactivity of the cell-free medium was determined.

The synthesis of agA in crown gall tumours was detected at 5 days incubation of 1 mg healthy tissue in 25 µl MS medium, supplemented with antibiotics and 2.5 µCi $^{32}$P-carrier-free phosphate pH 5.8 (see above). A sample of that extracellular liquid is mixed with an equal volume phenol and cleared by centrifugation. About 1−2 µl of the aqueous phase was spotted 5 cm from the cathodal side onto a thin layer of cellulose MN300, 20 x 20 cm. Electrophoresis was performed in a freshly made citrate buffer, 0.05 M, pH 5.0, 400 V (10−15 mA) for 1 h, in a Varsol-cooled flat-bed electrophoresis device as described previously by Ryler *et al.* (1955).

Positive and negative controls as well as an intracellular control and colour markers were included. The intracellular control was made by crushing thoroughly washed cells with an equal amount of pure phenol at 0°C. The aqueous phase, cleared by centrifugation, was used. The thin-layer plate was dried and subjected to autoradiography for at least 3 h. The mobility of agrocinopine (+ 0.32) is close to that of xylene cyanol FF, michrome 411, E. Gurr London (+0.30).

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