THE ISOLATION AND STRUCTURAL ELUCIDATION OF AGROCINOPINE C

A Thesis

submitted by

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to

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SUMMARY

Agrocinopine C and D are members of the most recently discovered class of opines, the Agrocinopines. These compounds are of interest, not only because of their opine nature, but also because they interact with the biological control agent of crown gall, Agrocin 84, altering its toxicity. In the present investigation Agrocinopine C has been isolated, and purified by a combination of anion and cation exchange chromatography and H.V.P.E. Degradative and physico-chemical studies have shown that Agrocinopine C consists of D-glucose-2-phosphate, linked in a phosphodiester bond to the sixth carbon of the glucose moiety of a sucrose molecule. Agrocinopine D is closely related, the only difference being the loss of the fructose moiety of the sucrose molecule.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University and to the best of my knowledge contains no material previously published or written by another person, except where due reference is made in the text.

A.E. Savage.

ACKNOWLEDGEMENTS

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1. INTRODUCTION

1.1. HISTORICAL BACKGROUND

Crown gall is a disease which affects many dicotyledonous plants, producing a cancerous growth on the crown and stem of the plant. The consequences of this growth range from an unsightly tumour of little practical importance to an interference with water and nutrient uptake causing stunting, and in severe cases death of the plant. In 1907 it was first recognised by Smith and Townsend that Crown gall was caused by a soil borne bacterium of the genus Agrobacterium.

The next major step in the study of Crown gall came in 1947 when Braun and White found that Crown gall tissue grew rapidly in tissue culture systems in the absence of any exogenous hormones and in the absence of the inciting bacterium. Normal plant tissue grows slowly in culture, and then only when plant hormones such as auxins and cytokinins are supplied. Braun (1947) concluded that the plant cells had somehow been transformed, or changed to tumour cells by infection with A.tumefaciens. He proposed that the bacterium introduced a "tumour inducing principle" (T.I.P.) into the plant cell. This hypothesis led to a search by many groups for the T.I.P.

Morel's group (1964) working in Versailles

provided an important metabolic clue to the nature of the T.I.P. They found that crown gall tissue synthesises a unique class of compounds which they called opines, and which are not found in normal plant tissue. The two compounds found were called octopine and nopaline. In 1968, Goldman showed that the opine produced was dependent on the strain of bacterium used to induce the tumour, and not on the nature of the host plant. It was subsequently found that those strains of bacterium that induced octopine type tumours could catabolise octopine, but could not catabolise nopaline, and conversely, those strains which induced tumours that synthesised nopaline could catabolise nopaline, but could not catabolise octopine (Petit 1970). These findings led Annik Petit to propose that the bacterium must insert into the plant cell a gene governing the synthesis of either octopine or nopaline, and that the tumour inducing principle must be DNA.

In 1969, Kerr reported that the property of virulence could be transferred from a virulent strain of Agrobacterium to an avirulent strain when two genetically marked strains were inoculated onto the same plant. This transfer took place even between distantly related Agrobacteria, making it unlikely that chromosomal genes were involved. These findings were confirmed in 1971, and Kerr concluded that the genes responsible for virulence were carried on some "transmissable element" such as a

bacterial virus or plasmid. Additional evidence that the genes for virulence were carried by a plasmid came from work by Van Larabeke (1974), who showed that incubation of Agrobacterium tumefaciens strain C58, at 37°C led to loss of virulence, as reported by Hamilton and Fall (1971) and in addition showed that this loss of virulence was associated with the loss of a large plasmid. In the same year Schell and Van Montagu (1974) found that the presence of a very large plasmid in Agrobacterium directly correlated with virulence. All the virulent strains which were studied contained this plasmid, while avirulent strains did not. Finally, Watson (1975) demonstrated that virulence transfer was due to the transfer of a large plasmid. This plasmid is now called the Ti plasmid or tumour inducing plasmid.

1.2. THE Ti PLASMID

The Ti plasmid has a molecular weight of approximately 120 x 10^6 daltons, giving it a coding capacity for about 200 average size proteins (Kerr 1978). In addition to virulence, several other properties have been attributed to the Ti plasmid, including the synthesis and catabolism of the opines. The significance of opines is embodied in the fact that Ti plasmids are now classified according to the family of opines for which they code catabolic functions in the bacterial cell. Thus at present there are three major classes of Ti plasmids which

code for catabolism of the imino acids known as octopine, nopaline and agropine.

Mutagenesis of the Ti plasmid by drug resistant transposon insertion (Hernalsteens 1978) and physical mapping by restriction endonuclease digestion has led to physical and genetic maps of the Ti plasmid (Chilton 1977, Depicker 1980). The Ti plasmid is responsible for many of the characteristics of Agrobacterium infection. These include:

(1) Oncogenicity (onc)

(2) Tumour formation (tum)

(3) Plasmid transfer (tra)

(4) Agrocin sensitivity, and

(5) Incompatibility (inc.)

1.3 T- DNA

Since Petit's hypothesis (1970) that the tumour inducing principle was in fact DNA, there have been many attempts to locate bacterial DNA in sterile crown gall tissue. Chilton and her co-workers (1977) were the first to convincingly detect a small segment of the Ti plasmid in crown gall tissue. This DNA sequence could not be found in normal plant tissue. Chilton's group compared the rate of reassociation of single stranded denatured Ti plasmid DNA, with the rate of its reassociation in the presence of crown gall DNA. They reasoned that if the tumour cell DNA included plasmid DNA, it

should add to the concentration of complementary strands, and so make the labelled plasmid DNA renature at a more rapid rate. Initial results showed no significant change in the reassociation rate. Clearly, therefore, there was not a copy of the entire Ti plasmid in the tumour tissue. The next approach, was to digest the labelled Ti plasmid with restriction endonucleases. When the renaturation experiments were repeated using individual DNA plasmid fragments, it was found that two of the fragments were renatured more rapidly in the presence of tumour DNA. Those two segments of the Ti plasmid must therefore be present in the tumour cell. This work has since been confirmed by the Belgium crown gall research group using ^{32}P labelled restriction fragments of the Ti plasmid as probes in southern blot hybridisation studies (Schell et al 1979). Subsequent experiments with many tumour lines have shown that specific fragments of the Ti plasmid are always present in the crown gall cells. (Chilton 1978, Depicker 1978). These specific fragments of DNA induce the tumorous state in crown gall disease. Chilton (1978) named these fragments transfer DNA or T-DNA.

Further evidence that T-DNA is transferred to the plant cell was provided by Schell et al (1979) and confirmed by Holsters (1980). Schell inserted the drug resistant transposon Tn 7 into various locations in the Ti plasmid. These mutants were used to induce tumours and it was found that one of

the mutants failed to synthesise nopaline. The transposon Tn7 was mapped and found in the T-DNA of the Ti plasmid. Using Southern blot techniques the transposon was also found in the sterile crown gall tissue. This work not only confirmed the hypothesis that DNA transfer had occurred, but also located the genes responsible for nopaline synthesis within the T-DNA. Even more exciting was the realisation that the transposon Tn 7 (a sizable piece of DNA) was present in the tumour cell. This meant that a large segment of foreign DNA, enough to carry several genes can be transferred along with the T-DNA. Thus the Ti plasmid was in theory, a potential vector for introducing desirable genes into plant cells.

A gene is expressed when its DNA is transcribed into messenger RNA (mRNA) and the RNA is translated into protein. Drummond et al (1977) showed that the T-DNA is transcribed into mRNA, and that this mRNA appears to have all the characteristics of eukaryotic mRNA rather than those of prokaryotic or bacterial mRNA.

The means by which T-DNA is transferred to the plant cell, and its location within the plant genome is unknown. Chilton (1980) analysed the DNA of the mitochondria and chloroplast for the presence of T-DNA, but found no evidence of its presence there. She concluded that the T-DNA was not located in known

plastid DNA, but was incorporated into nuclear DNA, or alternatively it was present in some as yet unidentified plastid DNA. Schell (1979) showed that tumour cell T-DNA extracted from plant cells was not completely homologous to probes made from the Ti plasmid. This indicated that the T-DNA is covalently linked to the plant DNA.

1.4 INSERTION OF FOREIGN GENES INTO PLANTS

The unique biological characteristics of the Ti plasmid make it a natural agent for gene transfer. A study of the structure of the T-DNA has revealed that the border fragments contain a 25 nucleotide "direct repeat" sequence of nucleotides. (Zambryski 1980). The precise repetition of short nucleotide sequences, either direct or inverted, often serves as a signal for recombinational events. It was this phenomenon which led two independent investigators to develop a "shuttle vector" in which a large part of the internal T-DNA has been deleted. (Leemans et al 1981, Chilton 1983). Foreign genes have been successfully introduced into plants, (Garfinkel et al 1981; Ooms P.J. 1980; Leemans et al 1981; Hernalsteens et al 1980) but at this stage the expression of these genes and the regeneration of healthy plants have not been reported in the same experiment.

1.5 BIOLOGICAL CONTROL

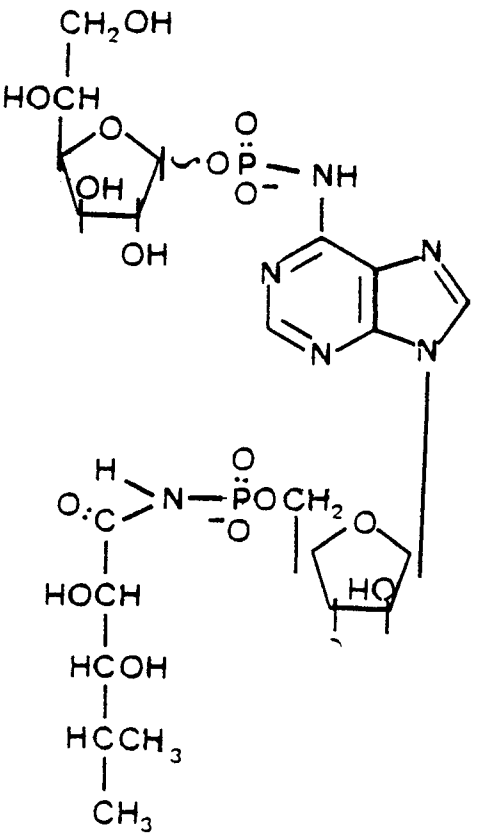
In the field, crown gall can cause quite serious crop losses. A successful biological control of some strains of Agrobacterium has been established (Kerr and Htay, 1974). The non pathogenic strain of Agrobacterium, K84, produces a highly specific bacteriocin, Agrocin 84. Sensitivity to this bacteriocin is a plasmid determined function. Most bacteria carrying the nopaline type Ti plasmids are sensitive to Agrocin 84. (Kerr and Roberts 1976).

A detailed analysis of the chemical structure of Agrocin 84 revealed that it is a disubstituted adenine nucleotide. (Figure 1.1). (Tate et al 1979). Tate also reported evidence which suggested that the 5' phosphoryl linkage from the fraudulent nucleotide core to the amide group is required for antibiotic activity, while the bacteriocin like specificity is due to a D-glucofuranosyl-oxyphosphoryl substituent at the N^6 of the adenine. Murphy and Roberts (1979) showed that the basis for sensitivity to Agrocin 84 resides in the presence of a high affinity uptake system encoded by the Ti plasmid. A nopaline Ti plasmid encoded permease found in the periplasmic space of bacteria harbouring the plasmid recognises the N^6 substituent of Agrocin 84, and selectively transports it into the cell. This molecule then behaves as an antibiotic, killing the pathogenic bacterium.

FIGURE 1.1 THE CHEMICAL STRUCTURE OF THE DI-SUBSTITUTED ADENINE NUCLEOTIDE AGROCIN 84.

The specificity is conferred by the D-glucofuranosyloxy-phosphoryl substituent at the N^6 of the adenine while the 5 phosphoryl linkage from the fraudulent nucleotide core is required for antibiotic activity.

AGROCIN 84


CH2OH
HOCH
O
OH
OH
O
P
O-
NH
N
N
N
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H
O
C
N
P
O
O-
CH2
O
HO
HOCH
HCOH
HCCH3
CH3

1.6 THE OPINES

1.6.1 The Opine Concept.

Four classes of opines have been described. These are the octopine family, the nopaline family, the agropine family, and the agrocinopines. (Menage and Morel 1964; Goldman et al 1969; Firmin and Fenwick 1978; Ellis and Murphy 1981). These unusual metabolites produced by crown gall tissue do not play a role in tumour induction or maintenance. This has been demonstrated by isolating mutants which no longer induce the synthesis of these substances, but still induce tumour formation. (Klapwijk et al 1978, Koekman et al 1979, Holsters et al 1980). Instead these metabolites appear to play a nutritional role for the bacteria, as they can be utilised by the Agrobacterium harbouring the Ti plasmid, as the sole source of carbon, nitrogen and phosphorous. The gene for nopaline synthesis has been mapped, and is located in the T-DNA portion of the pTiC58 plasmid (Holsters et al 1980), while the catabolic genes are located elsewhere on the Ti plasmid (Montoya et al 1977).

The opines have been defined as "compounds whose biosynthesis in crown gall tissue is directed by T-DNA and can be catabolised via Ti plasmid encoded pathways to supply a source of nutrients to Ti plasmid harbouring bacterium". (Petit et al 1978, Schell et al 1979). In this way, by transforming a single plant cell, the bacterium creates

a specific ecological niche for itself, rich in metabolites that it alone can readily catabolise, but are not readily available to the host plant or to other micro-organisms. The production of opines by crown gall tumours, at the direction of Ti plasmid borne genes, appears to be the biological rationale for the existence of the crown gall tumour. This model is at present challenged by only one group of plasmids, the "unusual nopaline plasmids", pTi AT 181, pTi EU9 and pTi T10/73, which confer nopaline utilisation on the host bacterium and elicit no opine production (Petit et al 1970, Sciaky et al 1978). These plasmids resemble one another closely and are very similar to pTi T37, a standard nopaline type Ti plasmid. They are thus viewed as "defective" or mutant nopaline type Ti plasmids (Guyon et al 1980). All other plasmids have been found to conform to the opine concept. In effect the Agrobacterium is seen to genetically manipulate the plant to its own benefit.

In addition to serving as nutrients, the opines act as specific inducers of conjugational transfer of Ti plasmids. (Petit et al 1978). The conjugative activity of the octopine Ti plasmid is promoted by preculture of the plasmid donor strain in the presence of the octopine opines. In the same way the conjugation of the nopaline type Ti plasmid is promoted by Agrocinopine A and B, and Agropine Ti plasmid transfer is induced by Agro-

cinopine C and D. Thus the opines promote the spread of the Ti plasmid to non pathogenic Agrobacteria, thus increasing the population of Agrobacteria carrying a specific Ti plasmid.

1.6.2 Opine Chemistry

As indicated, there are at present, four recognised families of opines: The Octopine family, the Nopaline family, the Agropine family and the Agrocinopines. Most Ti plasmids carry the genes for more than one family of opines and a summary of the opines so far found in transformed plant tissue can be seen in Table 1.1. The first three families of opines consist of N^2 substituted L-amino acids. The Octopine family consists of octopine, lysopine, histopine and octopinic acid. These are the condensation products of pyruvic acid and arginine, lysine, histidine and ornithine respectively (Figure 1.2). Nopaline and Nopalinic acid, the Nopaline family, are the reductive conjugates of α-ketoglutaric acid with arginine (in the case of nopaline) and ornithine (in the case of Nopalinic acid) (Fig. 1.3). The Agropine family consists of Agropine; the 1,2 lactone of deoxymannityl glutamine; deoxymannityl glutamine, a reductive conjugate of mannose and L-glutamine; and deoxymannityl glutamic acid, the reductive conjugate of mannose and L-glutamic acid. (Fig. 1.4).

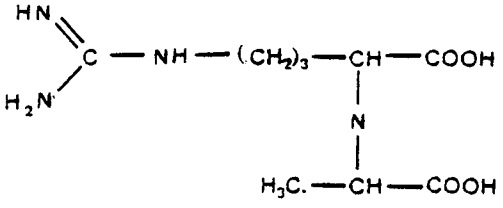
TABLE 1.1

OPINES IN TRANSFORMED PLANT TISSUE

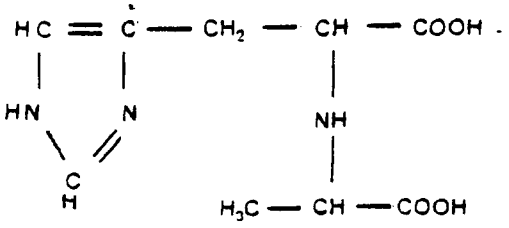
TYPE OF VIRULENCE PLASMID	EXAMPLES	OPINESIN PLANT TISSUE
Octopine Ti plasmids	B6, ACH5 R10	lysopine, Octopinic acid, Octopine histo- pine deoxymannityl- glutamine Agropine
Nopaline Ti Plasmids	C58, T37	Nopaline Nopalinic Acid Agrocinopines A & B
Agropine Ti Plasmids	Bo542, AT4	Deoxymannityl glutamine Deoxymannityl glutamic acid Agropine Agrocinopines C and D

FIGURE 1.2 The pyruvic acid derived opines; Octopine, octopinic acid, lysopine and histopine.

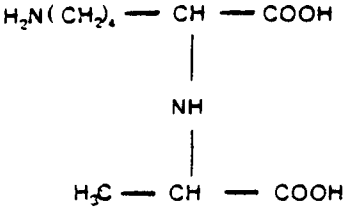
THE OCTOPINE FAMILY OF OPINES



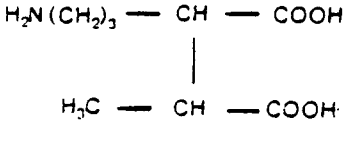
OCTOPINE



HISTOPINE



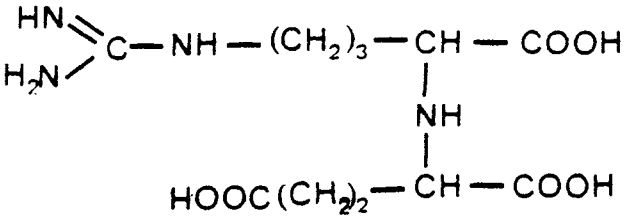
LYSOPINE



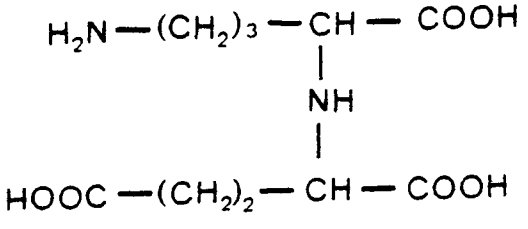
OCTOPINIC ACID

FIGURE 1.3 The ketoglutaric acid derived opines, nopaline and nopalinic acid.

THE NOPALINE FAMILY OF OPINES



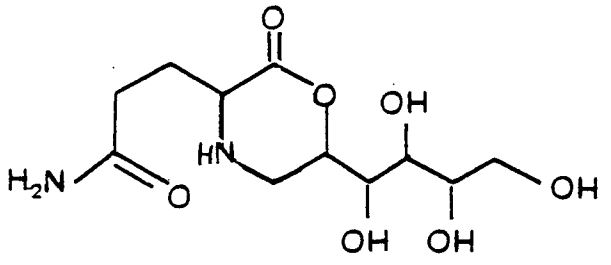
NOPALINE



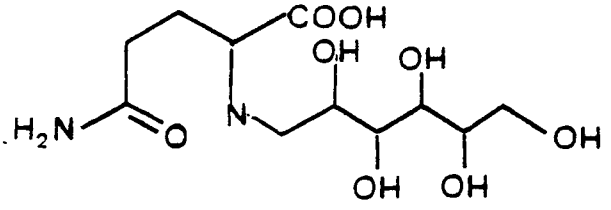
NOPALINIC ACID

FIGURE 1.4 The opines derived from the reductive conjugation of mannose and L-glutamine, Agropine, deoxymannityl glutamine and deoxymannityl glutamic acid.

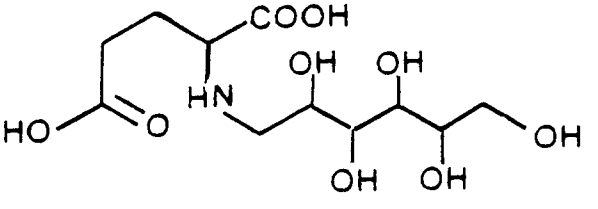
THE AGROPINE FAMILY



AGROPINE



DEOXYMANNITYL GLUTAMINE



DEOXYMANNITYL GLUTAMIC ACID

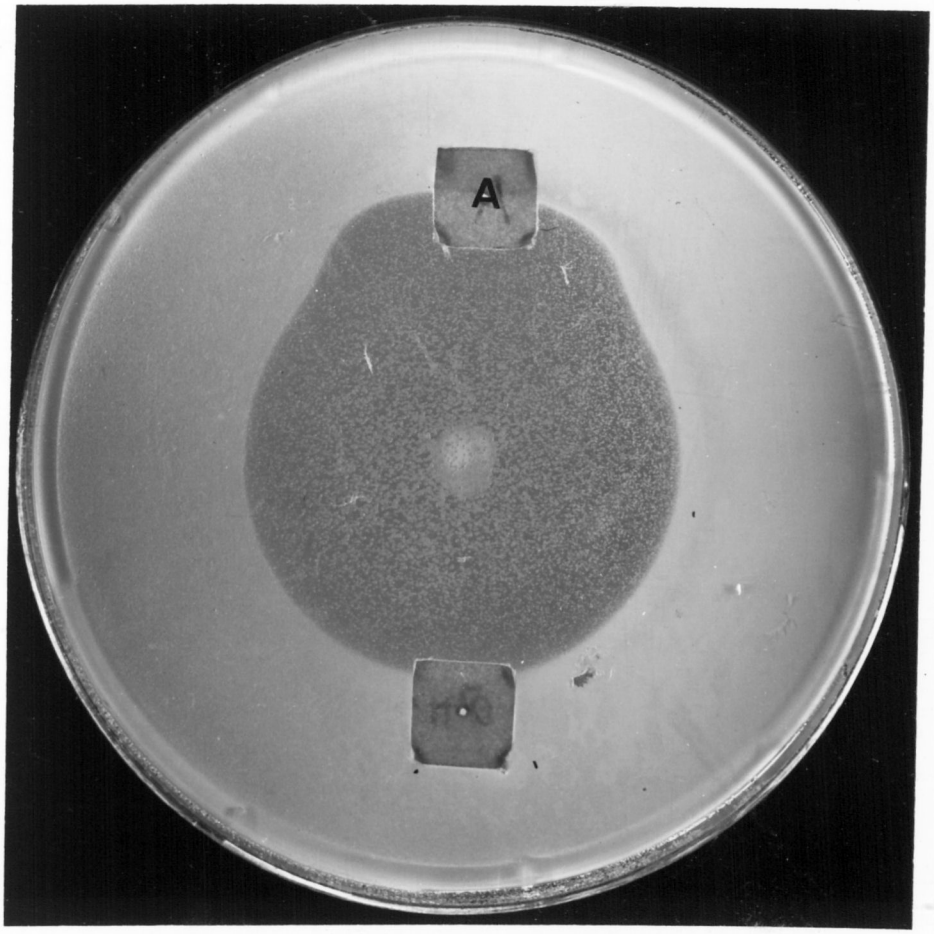
1.6.3 The Agrocinopines.

The Agrocinopines are the most recently discovered class of opines. (Ellis & Murphy 1981). Their recognition stemmed from the observation that sensitivity to the toxic compound Agrocin 84 was a plasmid-borne trait, and hence that this sensitivity pointed clearly to the presence of a permease in sensitive strains of Agrobacterium, which allowed the toxic compound Agrocin 84 to enter the bacterial cell as found by Murphy and Roberts (1979).

Ellis and Murphy (1981) reasoned that the primary function of this permease was probably the transport of some beneficial compound(s) into the bacterium, and they began searching for such compound(s). They found four compounds which not only interacted with Agrocin 84 activity, but also conformed with all the criteria characteristic of the opines. These compounds were called Agrocinopines.

Agrocinopine A and B are present in nopaline type tumours. They were found to increase the toxic effect of Agrocin 84 in the Stonier plate bioassay as reported by Kerr and Htay (1974) (Fig. 1.5). Agrocinopine C and D are found in Agropine type tumours. When tested in the same bioassay system Agrocinopine C counteracts the toxic effect of Agrocin 84. Further, when

FIGURE 1.5 BIOASSAY FOR AGROCINOPINE A.
Agrocinopine A is present in the square labelled A. As can be seen it increases the toxic effect of Agrocin 84 on the sensitive strain of Agrobacterium K476.


A

Agrocinopine C is tested in the plate bioassay system, with the Agrocin 84 insensitive strain K478 as the indicator strain, a zone of inhibition appeared where Agrocinopine C had been added. The biological activity of the Agrocinopines could not be detected in the absence of Agrocin 84.

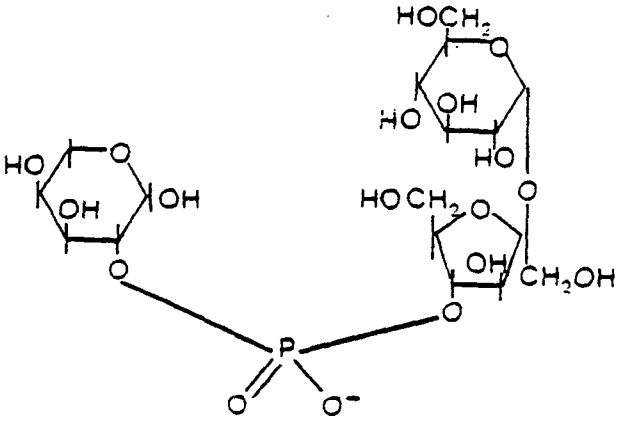
Preliminary qualitative work by Ellis and Murphy (1981) revealed that the agrocinopines were phosphorylated sugars. They are the only opines which contain phosphorous, and the only opines so far discovered which do not contain nitrogen. Results indicated that Agrocinopine A contains glucose, fructose, arabinose and phosphate, whilst Agrocinopine C contains glucose, fructose and phosphorous. Agrocinopine A and C can be degraded by mild acid hydrolysis to form Agrocinopine B and D respectively. This indicated that there was a close structural relationship between Agrocinopine A and B and Agrocinopine C and D.

The complete structures of Agrocinopine A and B have now been elucidated. (Ryder 1983). Agrocinopine A consists of an 1-arabinose -2-phosphate joined to the 4 hydroxyl of the fructose portion of sucrose via a phosphodiester linkage (Fig. 1.6). The determination of the structures of Agrocinopine C and D are the subject of this thesis.

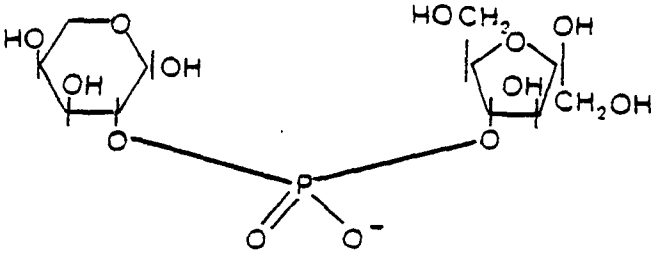
FIGURE 1.6 THE CHEMICAL STRUCTURES OF AGROCINOPINES A AND B.

Agrocinopine A consists of an arabinose-2-phosphate joined to the 4 hydroxyl of the fructose portion of sucrose. Agrocinopine B is closely related to Agrocinopine A, the only difference being the loss of a glucose molecule.

AGROCINOPINE A AND B



AGROCINOPINE A



AGROCINOPINE B

2. MATERIALS AND METHODS

2.1 MAINTENANCE OF CULTURES

Agrobacterium tumefaciens strains K478, K457, K476 and K84 were maintained at 4°C on yeast mannitol slopes (Appendix 1) in 25ml McCartney bottles. Subcultures of the appropriate strain were prepared on fresh yeast mannitol slopes and incubated for 3 days at 26°C before use.

2.2 DETECTION OF AGROCINOPINE C AND D AND RELATED COMPOUNDS

2.2.1 Bioassay for Agrocinopine C. (Ellis, 1980).

Agrobacterium strain K84 was inoculated onto the centre of Stonier's plates (Appendix 1) and incubated at 26°C for 36 hours. 1cm squares of Whatman No. 1 paper containing a suitable aliquot (~10 ul) of the solution to be tested were dried and placed in a circle (Diam. 2cm) surrounding the Agrocin 84 producing strain. The plates were then surface sterilized with chloroform vapour, overlaid with a soft buffered agar (Appendix 1) suspension of the indicator strain (K478 or K476), and incubated at 27°C for 2-3 days. An inhibition zone appears in the presence of Agrocinopine C and D. Negative Agrocin 84 controls were prepared by omitting the inoculation with *Agrobacterium* strain K84.

2.2.2 High Voltage Paper Electrophoresis (H.V.P.E.)

Samples (1-10ul) were applied as spots across the centre of Whatman 1M paper 57cm x 15cm. A standard solution containing Orange G, fructose, 2 deoxyadenosine and xylene cyanol was also loaded to provide running markers. The paper was then dipped in the appropriate buffer and placed in a perclean-cooled High Voltage Electrophoresis Unit (Tate 1968). The time allowed to run the paper and the voltage used was dependent on the buffer system.

2.2.2.1 Preparative H.V.P.E.

Partial purification of samples was obtained using H.V.P.E. The sample was loaded as a 10cm band on Whatman 1M paper. Reference standards solutions were spotted on either side of the band. The paper was then dipped in the appropriate buffer system and subjected to electrophoresis. The paper was then dried in cool air, guide strips (2cm) including the reference standards, were removed from the edges, and stained with the appropriate detection reagent to locate the sample position.

The sample band was cut out and eluted (5 x 50ul) by centrifuging with water or ammonia in an Eppendorf centrifuge.

2.2.2.2 Buffer systems commonly used in high voltage electrophoresis and conditions under which they are usually run.

(1) 0.75M formic/1.0M acetic pH 1.75.
Electrophoresis 70 v cm^{-1} (3000v) 15min.

(2) 0.5M Sodium Citrate buffer pH 6.5.
Electrophoresis: 35 v cm^{-1} (1500v) ½ hour.

(3) 0.1M ammonium bicarbonate buffer pH 9.2.
Electrophoresis 35 v cm^{-1} (1500v) 45 min.

(4) 0.2M Ammonium borate complexing buffer.
Electrophoresis: 35 v cm^{-1} (1500v) 45 min.

(5) Lanthanum nitrate complexing buffer pH 1.7.
0.2M $La(NO_3)_3$ was prepared by boiling La_2O_3(16.29g) in concentrated HNO_3 (15.7M 20ml). The syrupy product was diluted to 500mls with the formic acetic buffer pH 1.7.
Electrophoresis: 1000v 2 hours.

2.2.2.3 Detection reagents.

(1) Alkaline silver nitrate reagent (Trevelyan et al.1950).
$AgNO_3$ (2g) is dissolved in 20ml H_2O, and diluted to 1 litre with acetone.

The electrophoretogram is dipped, dried and redipped in ethanolic NaOH (10ml of 20% NaOH to 90ml ethanol). Electrophoretograms may be fixed in sodium metabisulphite (100g in 1 litre H_2O) and rinsed in water, before drying.

(2) p anisidine reagent (Hough et al 1950).

p anisidine HCl (5g) is dissolved in ethanol (475ml). To this, 0.5mg of sodium metabisulphite in 25ml of H_2O is added.

Papers are dipped, dried and heated at 110°C for 3-5 mins. Hexose sugars stain yellow, Pentose sugars stain pink.

(3) Phosphomolybdate reagent (Phosphate reagent) (Hough et al 1950).

$NaMoO_4$ (50g) is dissolved in water (250ml). To this, 1N HCl (500m) and 72% $HClO_4$ (210ml) are added. The solution is diluted 1:1 with water. 40ml of stock reagent is diluted in 160mls of acetone.

Electrophoretograms are dipped, dried, heated for 20 min. at 75°C and then irradiated with short wave U.V. light. Phosphates appear as blue spots.

(4) Urea phosphoric acid reagent (Ketose reagent) (E. Avela et al 1977).

Urea (5g) is dissolved in 80% phosphoric acid (22.5ml). The solution is made to a volume of 250ml with ethanol.

Papers are dipped, dried and heated at 110°C for 3 min. Ketose sugars stain blue.

(5) Triphenyl tetrazolium reagent (Trevelyan et al 1950).

Tetrazolium chloride monohydrate (0.20%) is dissolved in chloroform.

Papers are dipped, dried and redipped in ethanolic NaOH. Excess tetrazolium is removed by soaking in water for 20 minutes.

Reducing sugars stain pink.

2.2.2.4 Determination of relative mobility.

The mobility of samples was expressed as mobility relative to Orange G (RM_{OG}).

$$RM_{OG} = \frac{\text{Distance of the sample from the origin}}{\text{Distance of O.G. from the origin}}$$

The position of the origin was determined by including a non migrating marker in the reference standards. For the borate system, 2-deoxy-adenosine was used as the non migrating marker. For other buffers fructose was employed.

2.2.3 Paper Chromatography.

Paper chromatography was carried out on Whatman 1M paper in the descending mode. The solvent system used was propanol/H_2O/NH_4OH (7/2/1).

Mobilities were measured relative to the solvent front.

$$Rf = \frac{\text{distance between the origin and solvent front}}{\text{distance between origin and sample}}$$

2.3 PRODUCTION AND PURIFICATION OF AGROCINOPINE C AND D

2.3.1 Growth of Gall Tissue.

Tomato plants grown under glasshouse conditions (6-8 weeks old) were wounded and inoculated with Agrobacterium strain K478. Up to 3 inoculations were performed on the stem of each plant. The galls were allowed to develop for 6-8 weeks and were then removed from the plant.

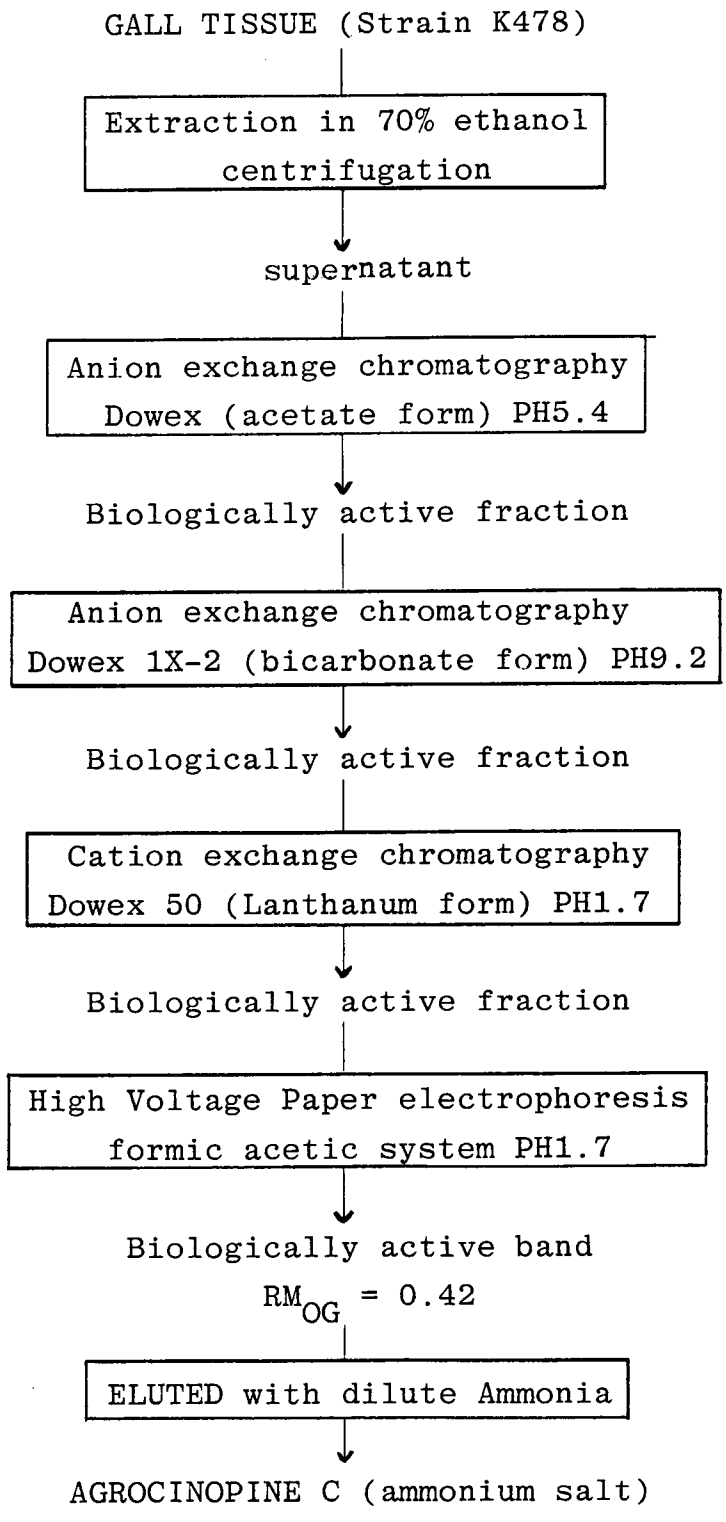
2.3.2 Extraction of Agrocinopine C from Gall Tissue.

Gall tissue was macerated in 70% ethanol using a Semak Vitamizer, followed by a kinematic Polytron K homogeniser. The material was then centrifuged in a Sorvall RC-5B refrigerated super speed centrifuge at 2310g for 15 minutes. The supernatant was collected and the pellet washed three times with 70% ethanol. The ethanol was removed in a rotary evaporator. Material which was insoluble in water formed a precipitate which was removed by filtration in a buchner funnel.

2.3.3 Purification.

The purification scheme can be seen in Figure 2.1.

FIGURE 2.1 PURIFICATION PROCEDURE



2.3.3.1 Anion exchange chromatography. (acetate form)

Dowex 1X-2 anion exchange (100gm) in the acetate form was suspended in distilled water (1 litre). The slurry was degassed before being packed into a column of dimensions 2.5cm x 30cm.

The crude ethanolic extract was loaded onto the column and washed with 2.5 litres of water. Agrocinopine C was displaced from the column using 0.2M pyridine 0.1M acetic acetic acid buffer. Four hundred 10ml fractions were collected. Those fractions showing biological activity were bulked, and dried by rotary evaporation at 30°C.

2.3.3.2 Anion exchange column chromatography. (bicarbonate form)

100g of Dowex 1X-2 (acetate form) was converted to the bicarbonate form by passing 0.2m ammonium bicarbonate through it in a Buchner funnel.

The dried eluate (from Dowex 1X-2 (acetate) column) was taken up in H_2O (300mls), loaded onto the column, and washed with 2 litres of H_2O. The column was eluted with a linear gradient of ammonium bicarbonate, pH 9.2, from 0 to 0.2m. Three hundred 10ml fractions were collected. Fractions showing biological activity were pooled, and dried on a rotary evaporator at 30°C.

2.3.3.3 Cation exchange chromatography.(Lanthanum form)

A 50ml column of Dowex 50W (H + form) was converted to the Lanthanum form by washing with $La(NO_3)_3$ until Lanthanum could be detected in the eluate. The column was then washed with water (500mls) until Lanthanum could no longer be detected in the eluate. The pooled dried Agrocinopine C sample was dissolved in a minimum amount of distilled water and layered onto the column. The material was eluted with water. 5ml fractions were collected. Agrocinopine C was detected by H.V.P.E. in a formic acetic and ammonium borate buffer system. This material was bulked and checked for biological activity.

2.3.3.4 H.V.P.E.

Eluate from the Dowex 50W column was dried on a rotary evaporator, taken up in a minimum amount of water (5mls), and loaded in a 10cm band onto Whatman 1M paper (200ul/paper). H.V.P.E. was performed for 30 min. at 1500 v in a formic acetic buffer system. The paper was dried and 2cm marker strips (including the standard and 3/4cm of the band) were cut off each side and stained to locate Agrocinopine C. The Agrocinopine C band was cut out and eluted with 0.01m ammonia solution. The eluates from each paper were pooled and lyophilised.

2.4 PHYSICO-CHEMICAL MEASUREMENTS

2.4.1 Infra-red Spectra

Infra-red spectra were recorded on a Perkin-Elmer Model 983 I.R. Spectrometer using slotted KC1 discs.

2.4.2 N.M.R. Spectra

The ^{13}C-N.M.R. Spectrum of Agrocinopine C (17.5mg) was recorded in D_2O (500ul) solution on a TEOL FX-100 instrument (with multi nuclear capacity). Courtesy of Dr. G. Jones, Plant Physiology.

The ^{13}C-N.M.R. Spectra of the standard compounds sucrose, glucose, glucose-1-phosphate and glucose-6-phosphate were also recorded for comparison with Agrocinopine C.

3(Trimethylsilyl) propionic acid-d_4 (TSP) was included in all samples as an internal reference standard. The ^{13}C-N.M.R. Spectrum of Agrocinopine A was kindly provided by Mr. M. Ryder.

2.5 TECHNIQUES RELATING TO DEGRADATIVE STUDIES

Degradative studies were followed by H.V.P.E. and on paper chromatography.

2.5.1 Acid Hydrolysis

Up to 200ul of sample and an equal volume of acetic acid (2m) were placed in a glass tube. The final concentration of acetic acid was 1m. The top of the tube was then sealed in a hot flame and incubated at 110°C. The time of hydrolysis was dependent on the extent of degradation required.

2.5.2 Ammonolysis

Up to 200ul of sample and an equal volume of ammonium solution (3m) were placed in a glass tube. The final concentration of ammonia was 1.5m. The tubes were again sealed in a hot flame and incubated at 110°C for 40 minutes.

2.5.3 Reduction

Sodium borohydride (2mg) was added to approximately 50ul of sample. The reaction was allowed to proceed for 30 minutes.

2.5.4 Enzymes

2.5.4.1 Glucose oxidase E.C.1134.

Type II glucose oxidase (β D-glucose : oxygen 1 oxidoreductase) from Aspergillus niger in the crystalline form was purchased from the Sigma Chemical Company. Specific activity 18 500 units/ gram solid.

Peroxidase E.C. 1.11:1.7 from horseradish type 1, 90 units/mg solid was used in the glucose oxidase buffer.

The glucose oxidase reaction mixture contained

1.035g $Na_2H\ PO_4\ \ 2H_2O$

0.545g $NaH_2\ PO_4\ \ 2H_2O$

224 U Peroxidase enzyme

and 7000 u glucose oxidase enzyme

made up to 75mls in distilled water.

10ul of this reagent was added to 20ul of sample. Incubations were carried out at room temperature for 2 hours.

2.5.4.2 Alkaline phosphatase E.C. 3131.

Alkaline phosphatase Type VII from calf intestinal mucosa as the crystalline suspension in 3.2m $(NH_4)_2SO_4$ solution was purchased from the Sigma Chemical Co.

1ul of enzyme was added to 20ul of sample.
The pH was checked with Neutralit pH indicator paper.
Incubations were carried out at 37°C for 2 hours.

2.5.5 Quantitative Assays.

2.5.5.1 Total phosphate determination (Bartlett 1959)

Bartlett's method of phosphate determination was used to determine total phosphorous content of a standard Agrocinopine C sample (18.24mg/ml).

A standard curve was prepared using 0-0.2 umoles of phosphate (K_2HPO_4).

2.5.5.2 Inorganic phosphate determination. (Murphy and Riley 1962).

Inorganic phosphate was measured using Murphy and Riley's method. A standard curve ranging from 0.004µmoles to 0.03µmoles of phosphate (K_2HPO_4) was prepared.

2.5.5.3 Total sugar content. (Dubois et al 1956)

The phenol-sulphuric acid colorimetric method for the determination of sugar concentration was used. It was necessary to perform strong acid hydrolysis (1m acetic acid 24 hrs. 110°C) before proceeding with the assay. A standard curve was prepared using D-glucose ranging from 0 to 0.40 umoles.

2.5.5.4 Glucose determination. (Bergmeyer 1971)

The total D-glucose concentration was also determined for Agrocinopine C. Again strong acid hydrolysis (1m acetic acid 110°C 24 hrs.) preceded the measurements. The method used involved the detection of D-glucose by measuring its oxidation by D-glucose oxidase. Hydrogen peroxide, a by-product of this reaction reacts with ABTS (2,2'-azino-di (3 ethyl benzthazoline sulphonic acid))

producing a green colour. Absorbance is read at 440nm. A standard curve was constructed using a D-glucose standard from 0umoles to 0.028umoles.

2.6 SYNTHESIS OF THE GLUCOSE PHOSPHATE STANDARDS

Glucose-1-phosphate and glucose-6-phosphate as sodium salts were purchased from the Sigma Chemical Co.

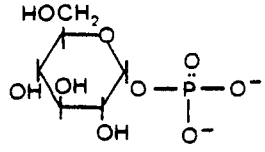
2.6.1 Glucose-2-Phosphate (G-2-P).

The sequence of reactions in the synthesis of G-2-P can be seen in Figure 2.2.

D-glucose-1-phosphate (G-1-P) Na^+salt (30mg) was passed through a 2ml Dowex 50 cation exchange column (H^+ form). G-1-P was eluted with distilled water (10mls) and pyridine (6mls) was added to the sample, which was then dried on a rotary evaporator at 40°C and taken up in pyridine (3mls). This yielded G-1-P as the pyridinium salt. The cyclising agent dicyclohexyl carbodiimide (D.C.C. (2mg)) was added, and the reaction incubated at 110°C for 40 minutes. Under these conditions the cyclic glucose-1,-2,-phosphate is formed. Excess D.C.C. was removed by precipitation with distilled water (10mls). The sample was dried on a rotary evaporator at 40°C and the water soluble material taken up in a minimum amount of distilled water (2mls). A 200ul aliquot

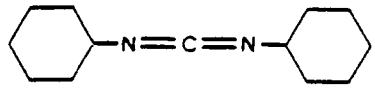
FIGURE 2.2 The reaction sequence used in the synthesis of glucose-2-phosphate.

SYNTHESIS OF GLUCOSE-2-PHOSPHATE

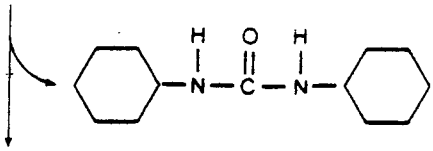


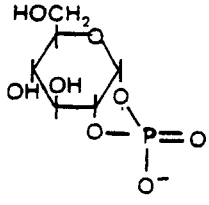
GLUCOSE 1 PHOSPHATE

+



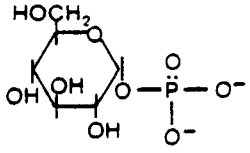
DICYCLOHEXYL CARBODIIMIDE





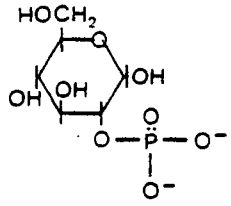
CYCLIC GLUCOSE 1,2 PHOSPHATE

weak acid hydrolysis



GLUCOSE 1 PHOSPHATE

+



GLUCOSE 2 PHOSPHATE

+ GLUCOSE

+ Pi

of this sample was hydrolysed with 1m acetic acid (equal volumes of 2m acetic acid) for 30 min. This yielded a mixture of the G-1-P, the G-2-P, and small amounts of glucose and inorganic phosphate.

2.6.2 Glucose-4-Phosphate (G-4-P)

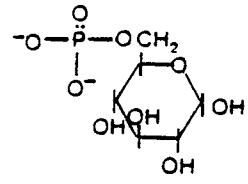
The sequence of reactions used to synthesise glucose-4-phosphate can be seen in Figure 2.3. Glucose-4-phosphate was synthesised using the same principle as was used in the synthesis of glucose-2-phosphate. In this case glucose-6-phosphate (pyridinium salt) was treated with D.C.C. to yield the cyclic glucose 4, 6, phosphate. This compound is much more stable than the cyclic glucose 1, 2 phosphate and stronger acid hydrolysis (0.5NHCl 110^{o}C 40 minutes) was required to cleave the cyclic phosphate. The end products were G-6-P, G-4-P, and a small amount of glucose and inorganic phosphate.

2.6.3 Glucose-3-Phosphate.(G-3-P)

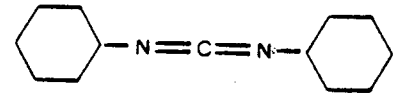
Glucose-3-phosphate was synthesised by Dr. M.E. Tate using a slightly modified version of the method outlined by Brown, Hayes and Todd (1957). The reaction scheme (Brown et al 1957) for the synthesis of glucose-3-phosphate can be seen in Figure 2.4.

FIGURE 2.3 The reaction sequence used in the synthesis of glucose-4-phosphate.

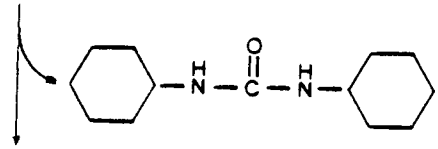
SYNTHESIS OF GLUCOSE-4-PHOSPHATE

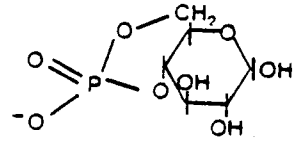


GLUCOSE 6 PHOSPHATE



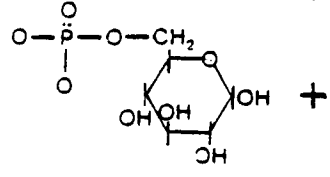
DICYCLOHEXYL CARBODIIMIDE



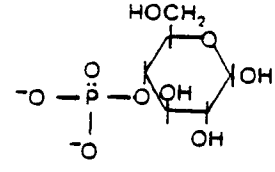


CYCLIC GLUCOSE 4,6-PHOSPHATE

strong acid hydrolysis



GLUCOSE 6 PHOSPHATE



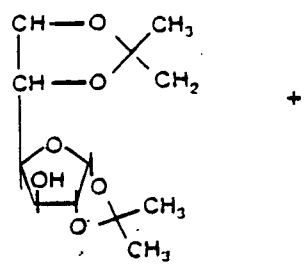
GLUCOSE 4 PHOSPHATE

+ GLUCOSE

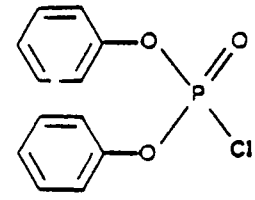
+ Pi

FIGURE 2.4 The reaction sequence used in the synthesis of glucose-3-phosphate.

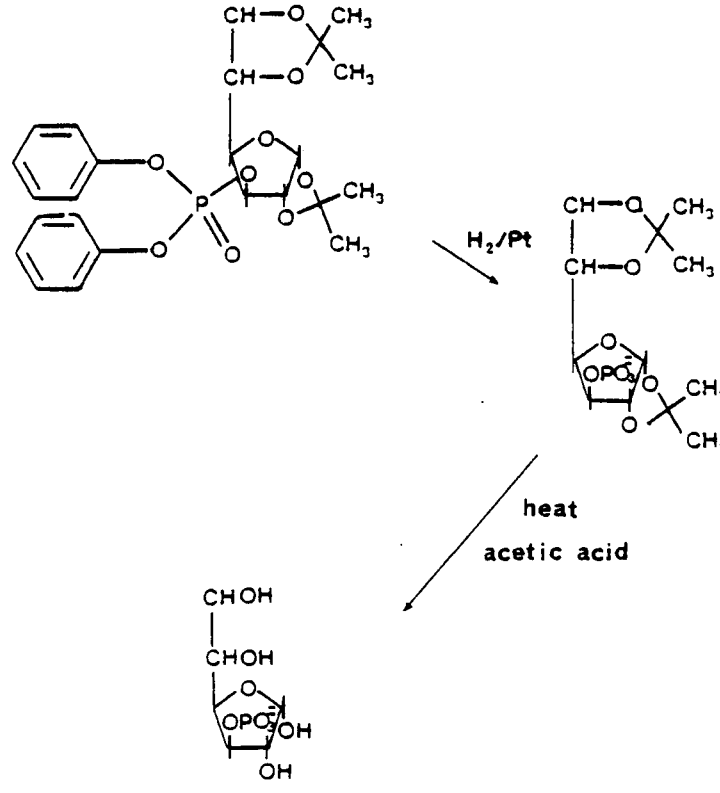
SYNTHESIS OF GLUCOSE-3- PHOSPHATE



1, ;5,6 DISOPROPYLIDENE GLUCOSE



DIPHENYLPHOSPHORO CHLORIDATE



GLUCOSE-3-PHOSPHATE

2.6.4 Purification of Glucose Phosphate Standards.

The glucose phosphates were partially purified by H.V.P.E. at pH 9.2 in an ammonium borate complexing system. The eluate from 4 papers (each containing a 10cm band of reaction mixture) was passed through a Dowex 50w (H^+) cation exchange column and eluted with 10mls of distilled water. This material was dried on a rotary evaporator at 40°C and redissolved in methanol (2mls). The methylborate complex is volatile and thus the borate was removed by evaporation to dryness, on a Savant speed vacuum centrifuge (Model No.SUC 100H).

Electrophoretically homogeneous samples of glucose-2-phosphate (2.45mg), glucose-4-phosphate (1.98mg) and glucose-3-phosphate (2.01mg) were thus obtained and were used as 1% solutions for electrophoretic and chromatographic reference standards.

3. RESULTS

3.1 BIOASSAY

Figure 3.1 shows the Agrocinopine C bioassay. The zone width is measured from the centre of the paper square inward to the edge of the inhibition zone. Agrocinopine C induces sensitivity to Agrocin 84 in the normally insensitive strain K478.

3.2 PURIFICATION OF AGROCINOPINE C

3.2.1 Dowex 1X-2 Column (acetate form)

Table 3.1 and Figure 3.2 show the biological activity in fractions eluted from the Dowex 1X-2 anion exchange column in the acetate form. Electrophoretic mobilities of components present in the biologically active material are listed in Table 3.2. Agrocinopine C has an RM_{OG} = 0.42 and reacts positively with p anisidine, urea phosphoric, silver nitrate and phosphate reagents (refer to Section 3.3.1). In addition to Agrocinopine C two cationic impurities were present as well as non migrating and anionic materials.

FIGURE 3.1 BIOASSAY OF AGROCINOPINE C.

Agrocinopine C is present in the paper squares marked C.

Agrocinopine C induces the normally insensitive strain K478 to become sensitive to Agrocin 84.

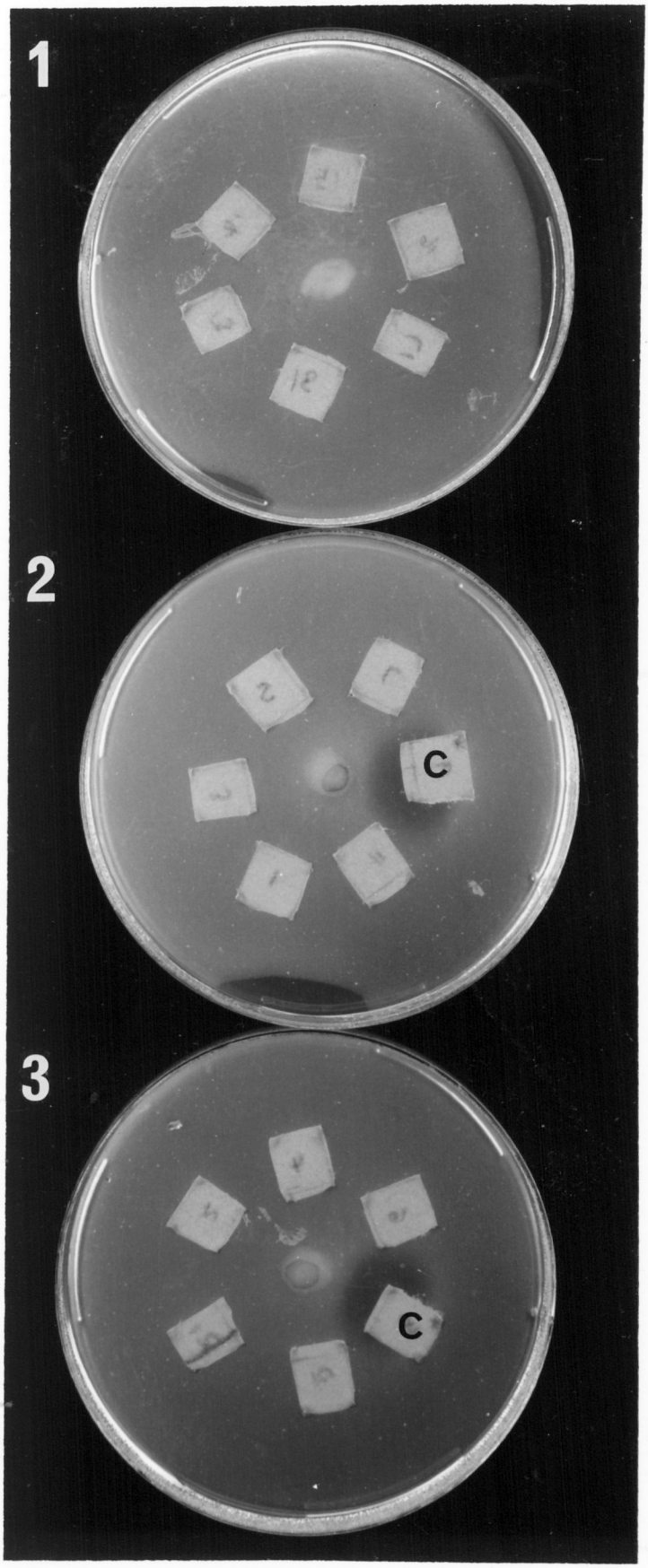

1
2
C
3
C

FIGURE 3.2 BIOLOGICAL ACTIVITY OF FRACTIONS FROM THE DOWEX 1X-2 (ACETATE) COLUMN.

Eluant: 0.2M pyridine/0.1M/acetic acid.
Column size: 500mls
fraction size: 10mls
Temperature: 5°C.

Agrocinopine C is present in fractions 100-240.

FRACTIONS FROM DOWEX 1x-2(ACETATE)

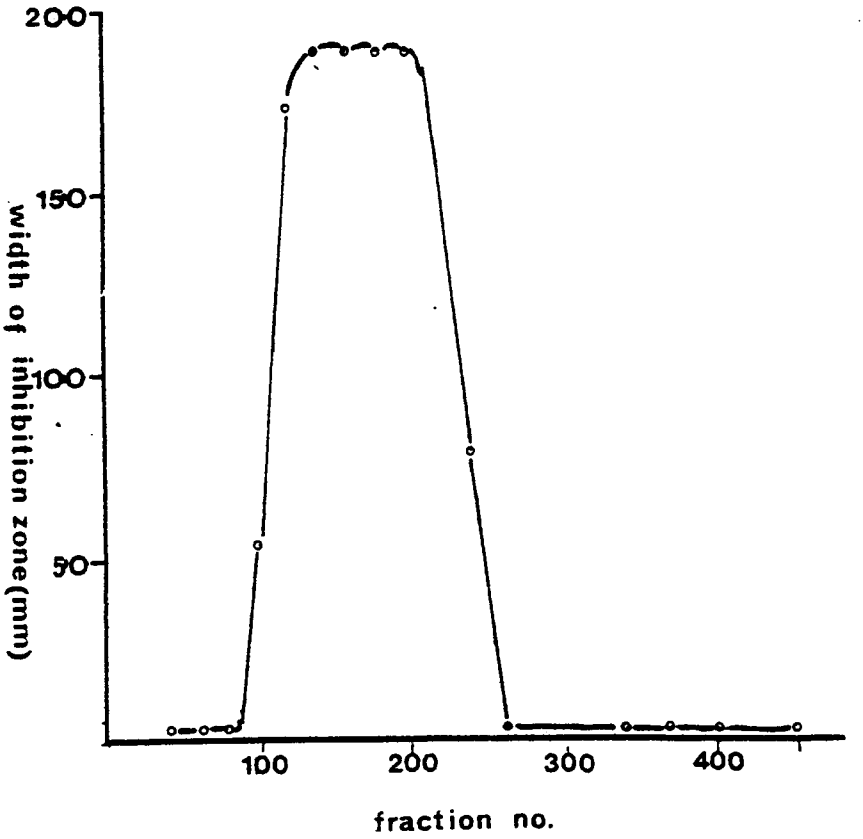


TABLE 3.1 *Biological activity of fractions from Dowex 1X-2 column (acetate form)*

Fraction No.	Width of inhibition Zone (mm)
40	0
60	0
80	0
100	10.4
120	18
140	19
160	19
180	19
200	19
220	19
260	12
240	8
300	0
340	0
360	0
380	0
400	0

TABLE 3.2 *Electrophoretic mobilities of the biologically active fractions of Dowex 1X-2 column (acetate form)*

RM_{OG}	Staining			
	A	B	C	D
-0.44	+	-	-	-
-0.40	+	-	-	-
0	+	+	-	-
0.32	+	-	-	-
0.42	+	+	+	+

Stain A = silver nitrate
Stain B = (urea - phosphoric acid) reagent
Stain C = p anisidine
Stain D = Phosphate reagent

3.2.2 Dowex 1X-2 Column (bicarbonate form)

Table 3.3 shows the biological activity in fractions eluted from the Dowex 1X-2 column in the bicarbonate form. The relationship between the fraction number, the biological activity and the ammonium bicarbonate concentration gradient used to elute the column is illustrated in Figure 3.3. Agrocinopine C was eluted from the column at an ammonium bicarbonate concentration ranging from 0.8m to 0.11m. The electrophoretic data (Table 3.4) for the biologically active material shows that some cationic and neutral impurities are still present.

TABLE 3.3 *Biological activity of fractions eluted from the Dowex 1X-2 anion exchange column (bicarbonate form)*

Fraction No.	Width of inhibition Zone (mm)
40	0
80	0
120	0
130	6.0
140	16.0
150	18
160	14
170	12
180	5
200	0
240	0
280	0
320	0
400	0

FIGURE 3.3 BIOLOGICAL ACTIVITY OF FRACTIONS FROM THE DOWEX 1X-2 (BICARBONATE COLUMN).

Eluant: Ammonium bicarbonate (0-0.2m)
Column size: 500mls
Fraction size: 10mls.

The left hand axis shows the width of the inhibition zone, while the axis on the right indicates the concentration of the Ammonium bicarbonate gradient.

_____ width of inhibition zone
-------- concentration of the Ammonium bicarbonate gradient.

Agrocinopine C is present in fractions 130-180.

FRACTIONS FROM DOWEX 1x-2 (BICARBONATE)

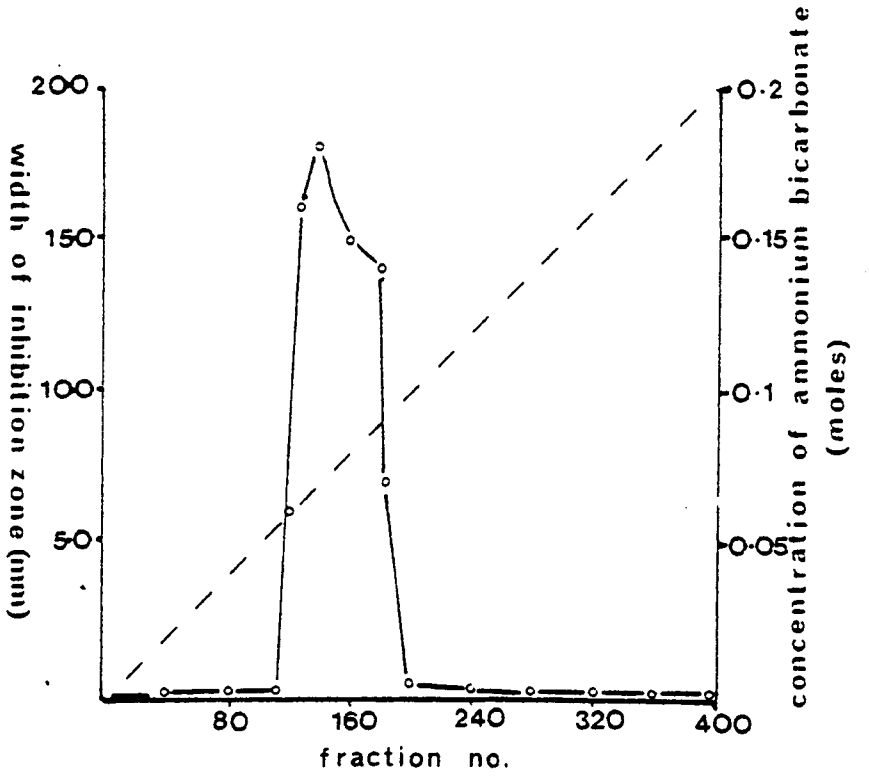


TABLE 3.4 *Electrophoretic mobilities of biologically active eluate from the Dowex 1X-2 column (bicarbonate form)*

RM_{OG}	Staining			
	A	B	C	D
0.45	+	+	+	+
0.04	+	+	-	-
-0.30	+	-	-	-

Stain A = silver nitrate
B = urea phosphoric acid reagent
C = p anisidine
D = phosphate reagent

3.2.3 Cation exchange Column (Lanthanum form)

Table 3.5 shows the mobility relative to orange G of material eluted from the Dowex 50 (Lanthanum) column. Fractions 2, 3 and 4 contained biological activity and were combined. Cationic impurities were retarded on the La^{2+} cation exchange column and hence were separated from Agrocinopine C.

3.2.4 Preparative H.V.P.E.

Agrocinopine C was detected in the preparative H.V.P.E. by its mobility relative to Orange G as well as its characteristic staining properties. Eluted material was bulked and the presence of biological activity confirmed.

TABLE 3.5 *Electrophoretic mobilities of Dowex 50 (lanthanum) column eluate*

Fraction No.	RM_{OG} 1	2	3	Staining A	B	C	D
2	0.42	0.43	0.42	+	+	+	+
	0	0.42	0.43	+	-	-	-
4	0.42	0.43	0.42	+	+	+	+
	0	0.42	0.43	+	-	-	-
6	0	0.42	0.43	+	-	-	-
8	0	0.42	0.43	+	-	-	-
10	0	0.42	0.43	+	-	-	-

Buffer system 1 = formic/acetic pH 1.7
2 = ammonium bicarbonate pH 9.2
3 = ammonium borate pH 9.2

Stain A = silver nitrate
B = ketose reagent
C = p anisidine
D = phosphate reagent

3.2.5 Yield of Agrocinopine C.

	% of the original fresh weight
311.78g gall tissue	
↓ Dowex 1X-2 (acetate)	
590.25mg	0.189
↓ Dowex 1X-2 (bicarbonate)	
340.30mg	0.109
↓ Dowex 50 (lanthanum)	
157.84mg	0.051
↓ electrophoresis (formic/acetic buffer pH 1.7)	
57.33mg of Agrocinopine C (ammonium salt)	0.018

3.3 STUDIES ON THE INTACT AGROCINOPINE

3.3.1 Staining Properties of Agrocinopine C and D

TABLE 3.6 *Staining characteristics of Agrocinopines C and D*

Stain	Agrocinopine C	Agrocinopine D
Silver nitrate (vicinal glycols)	+	+
p anisidine (Aldose & ketose)	+	+
Phosphate reagent (P)	+	+
Urea-phosphoric reagent (ketose)	+	-
Triphenyl Tetrazolium reagent (Enediols)	-	-
U.V. absorption (Conjugation)	-	-

3.3.2 Electrophoretic pH Mobility Profile: Charge Characteristics.

Courtesy of Mr. Ryder (unpublished data).

Purified Agrocinopines C and D were subjected to H.V.P.E. in buffers of varying pH (Tate 1968). Table 3.7 shows the results. Ribose-5-phosphate and bis-(p nitrophenyl) phosphate were used as standards to illustrate the behaviour of the phosphomonoesters and phosphodiesters respectively. Figure 3.4 shows that the phosphodiester bis-(p-nitrophenyl) phosphate has a flat pH mobility profile as do Agrocinopine C and D. Ribose-5-phosphate, the phosphate monoester undergoes two

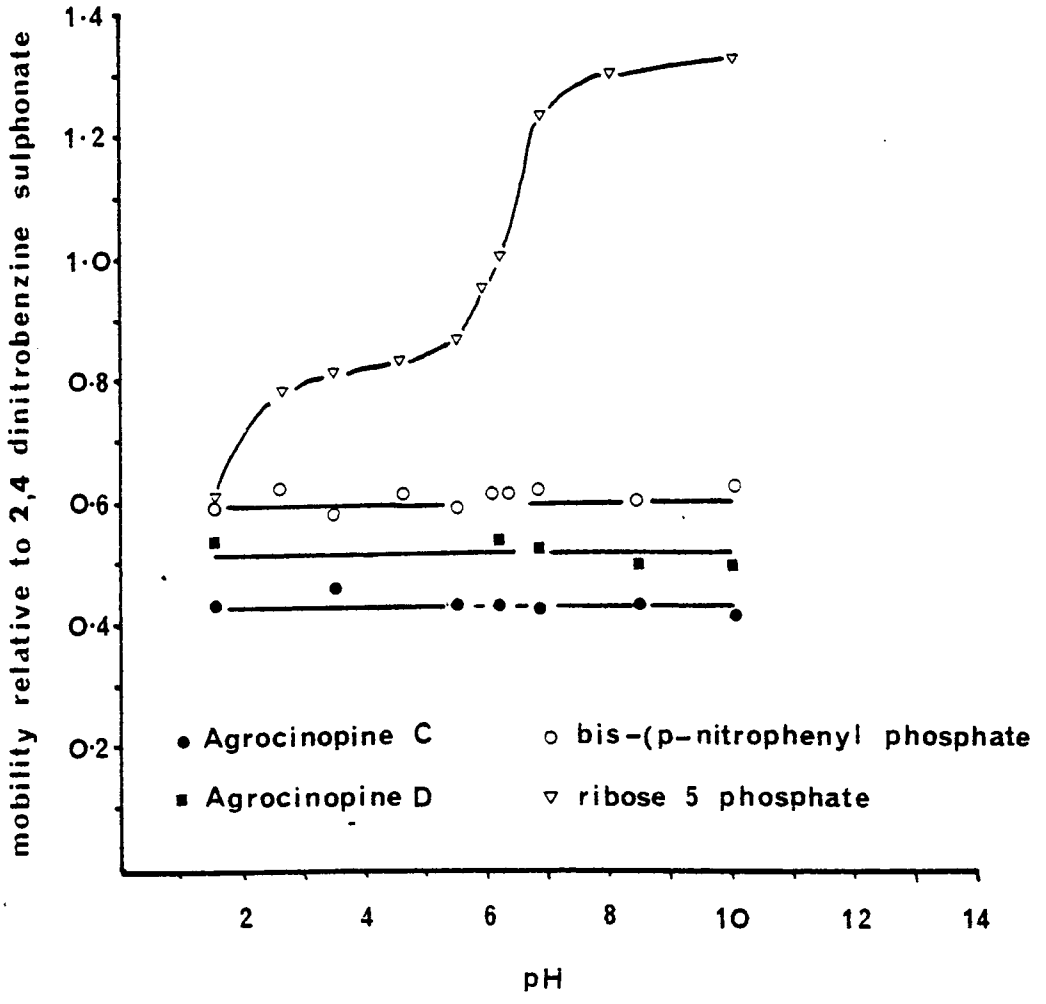
FIGURE 3.4 THE ELECTROPHORETIC pH MOBILITY PROFILE OF AGROCINOPINE C AND D.

The phosphodiester bis-(p nitrophenyl) phosphate and the phosphate monoester ribose-5-phosphate are included as standards for comparison.

Note: The mobilities are measured relative to 1, 2, 4 dinitrobenzene sulphonate.

pH MOBILITY PROFILE

AGROCINOPINE C AND D



dissociation steps (pK ~2 & pK ~6), as shown by its pH mobility profile.

TABLE 3.7 (Courtesy of M. Ryder)

pH Mobility profile of Agrocinopine C and D
Mobility relative to 2, 4, dinitrobenzene sulphonate

pH	Agrocinopine C	Agrocinopine D	Standard 1	Standard 2
1.49	0.44	0.53	0.59	0.59
2.6			0.62	0.78
3.7	0.46		0.58	0.81
4.62			0.61	0.83
5.54	0.43		0.59	0.86
5.96			0.61	0.95
6.56	0.43	0.55	0.61	1.00
6.98	0.43	0.52	0.62	1.23
8.46	0.43	0.49	0.60	1.30
9.95	0.41	0.49	0.53	1.32

Standard 1 = bis-(p-nitrophenyl) phosphate
2 = ribose-5-phosphate

3.3.2.1 Electrophoretic mobilities in the complexing buffers.

The mobility relative to Orange G of Agrocinopine C in the Ammonium borate and Lanthanum nitrate complexing buffers were also measured. In the Ammonium borate buffer pH 9.2 Agrocinopine C has a RM_{OG} = 0.42 and in the Lanthanum nitrate buffer pH 1.7 Agrocinopine C has a RM_{OG} = -0.44.

3.3.3 Paper Chromatography

Partially purified Agrocinopine C was located on paper chromatography by staining with the silver

nitrate reagent and the phosphate reagent. The solvent system used was Propanol : H_2O : NH_4OH (7 : 2 : 1). Agrocinopine C has a mobility relative to the solvent front Rf = 0.48.

3.3.4 Infrared Spectra

Figure 3.5 shows the infrared spectrum of Agrocinopine C from 400cm^{-1} to 4000cm^{-1}. Also shown are the spectra for the standard compounds glucose-2-phosphate and sucrose. Figure 3.6 compares the infrared spectrum of Agrocinopine C and Agrocinopine A.

3.3.5 ^{13}C-N.M.R. Spectrometry.

The ^{13}C-N.M.R. spectra are shown in Figures 3.7, 3.8 and 3.9. The carbon assignments are shown above the various peaks. Those peaks of importance which appear in more than one spectrum are linked by a dotted line.

Figure 3.7 shows the ^{13}C-N.M.R. spectrum of the 3 standard compounds glucose-6-phosphate, glucose and glucose-1-phosphate. Table 3.8 gives the corresponding chemical shift values (ppm). Figure 3.8 shows the ^{13}C-N.M.R. spectrum of Agrocinopine C, as well as that of the standard compounds glucose and sucrose. The chemical shift values (ppm) and the Jp-o-c values (Hz) are shown in Table 3.9. The final ^{13}C-N.M.R. spectrum is

FIGURE 3.5 THE INFRARED SPECTRUM OF AGROCINOPINE C.

The I.R. Spectrum of Glucose-2-phosphate and sucrose are included for comparison.

Concentration: 100ug in 50mg KC1.
Solvent: KC1 disc.
Reference: KC1.

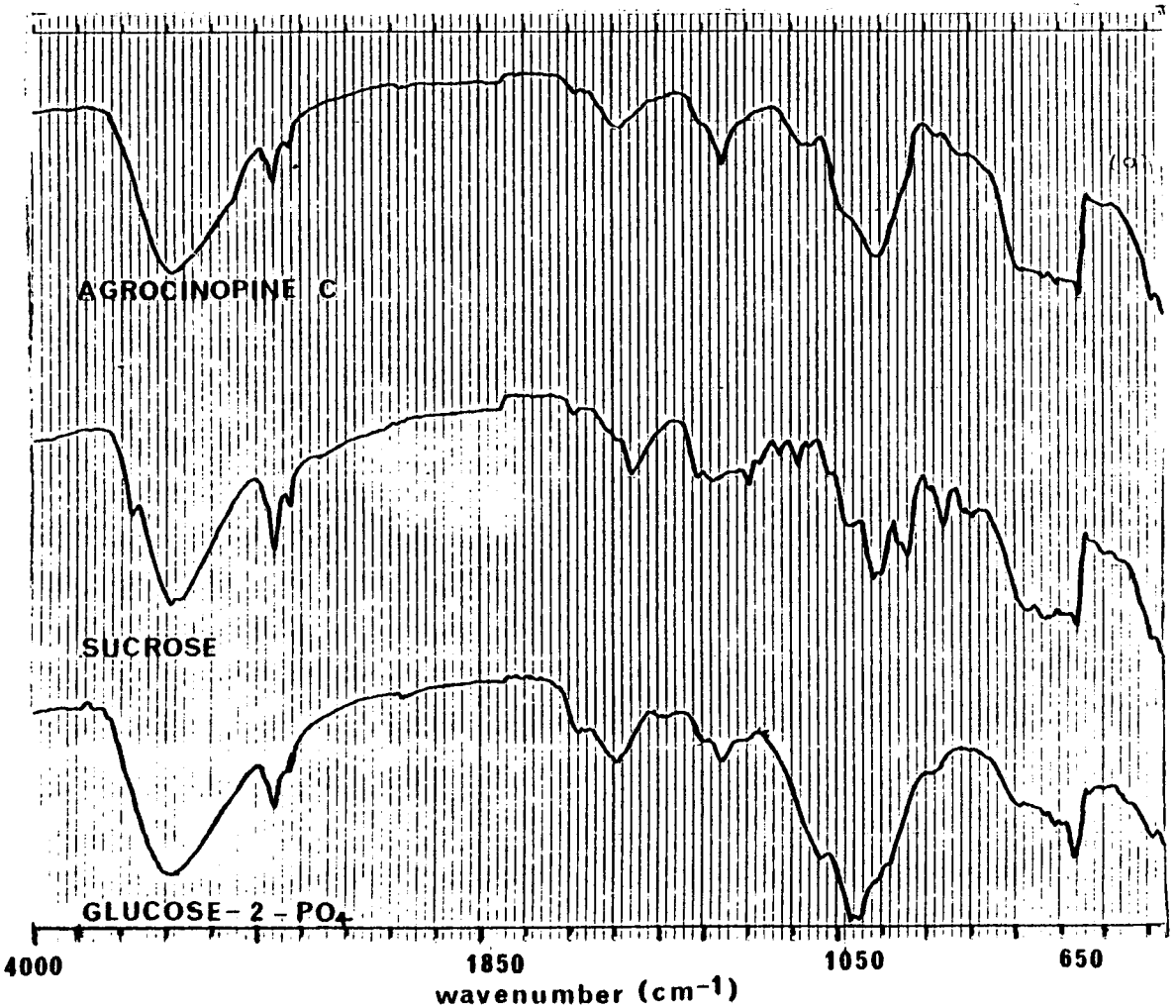

AGROCINOPINE C
SUCROSE
GLUCOSE-2-PO4
4000
1850
1050
650
wavenumber (cm-1)

FIGURE 3.6 COMPARISON OF I.R. SPECTRUM OF AGROCINOPINE A AND AGROCINOPINE C.

Concentration: 100ug in 50mg KC1
Solvent: KC1 disc
Reference: KC1.

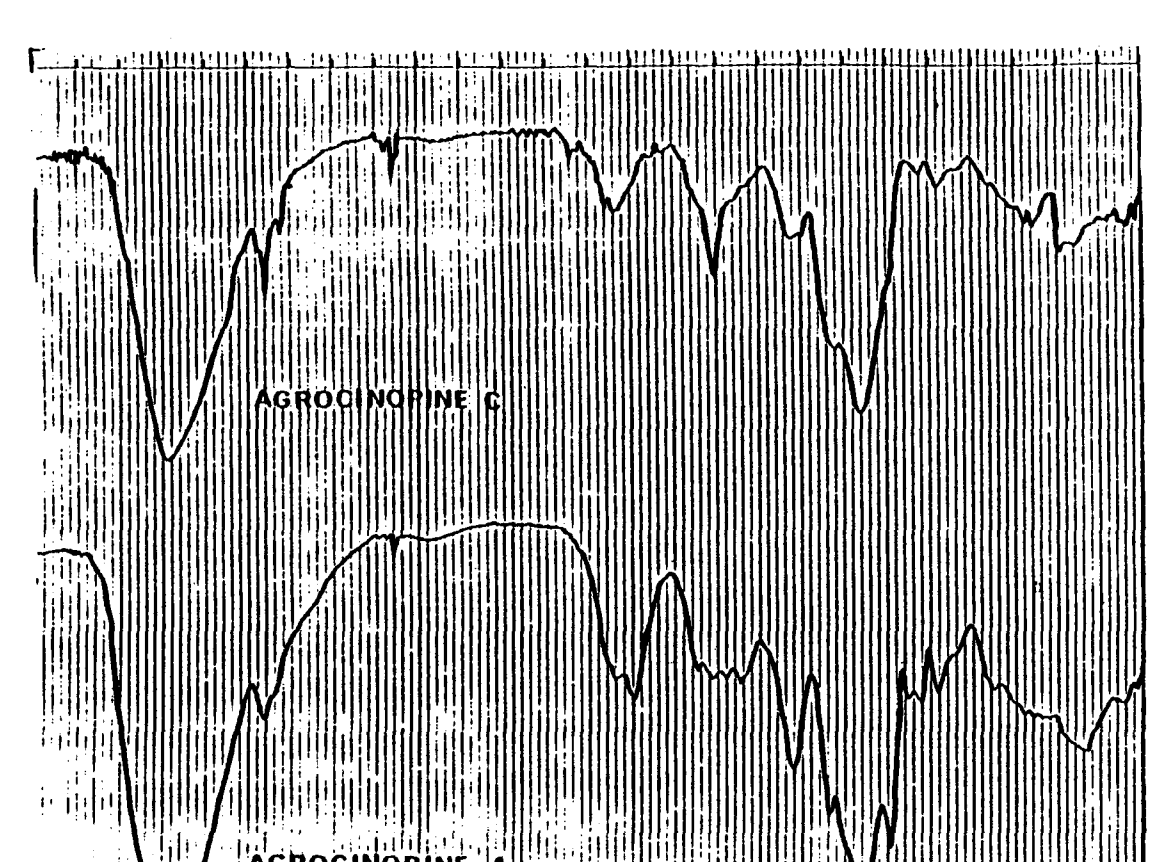

AGROCINOPINE C
AGROCINOPINE A
4000
1850
1050
650
wavenumber (cm-1)

FIGURE 3.7 ^{13}C NMR SPECTRA OF GLUCOSE-6-PHOSPHATE, GLUCOSE-1-PHOSPHATE AND GLUCOSE.

Peaks which appear in more than one spectrum are joined by a dotted line. The carbon assignments are presented above each peak.

Conditions of NMR spectrum.

Solvent: D_2O
Conc^n: 40mg/0.5ml.
Reference: T.S.P.
Nucleus: ^{13}C.
Frequency: 22.49 MHZ
Offset: 43.2 KHZ

Pulse width 11 usec.
Pulse delay 2 sec.
Pulse angle 45
Data points 8K/4K
Window 10.
No. pulses 730
Spectral amplitude 23 x 3db
Spectral width 5kHZ
Decoupling mode BB
Temperature 24^oC

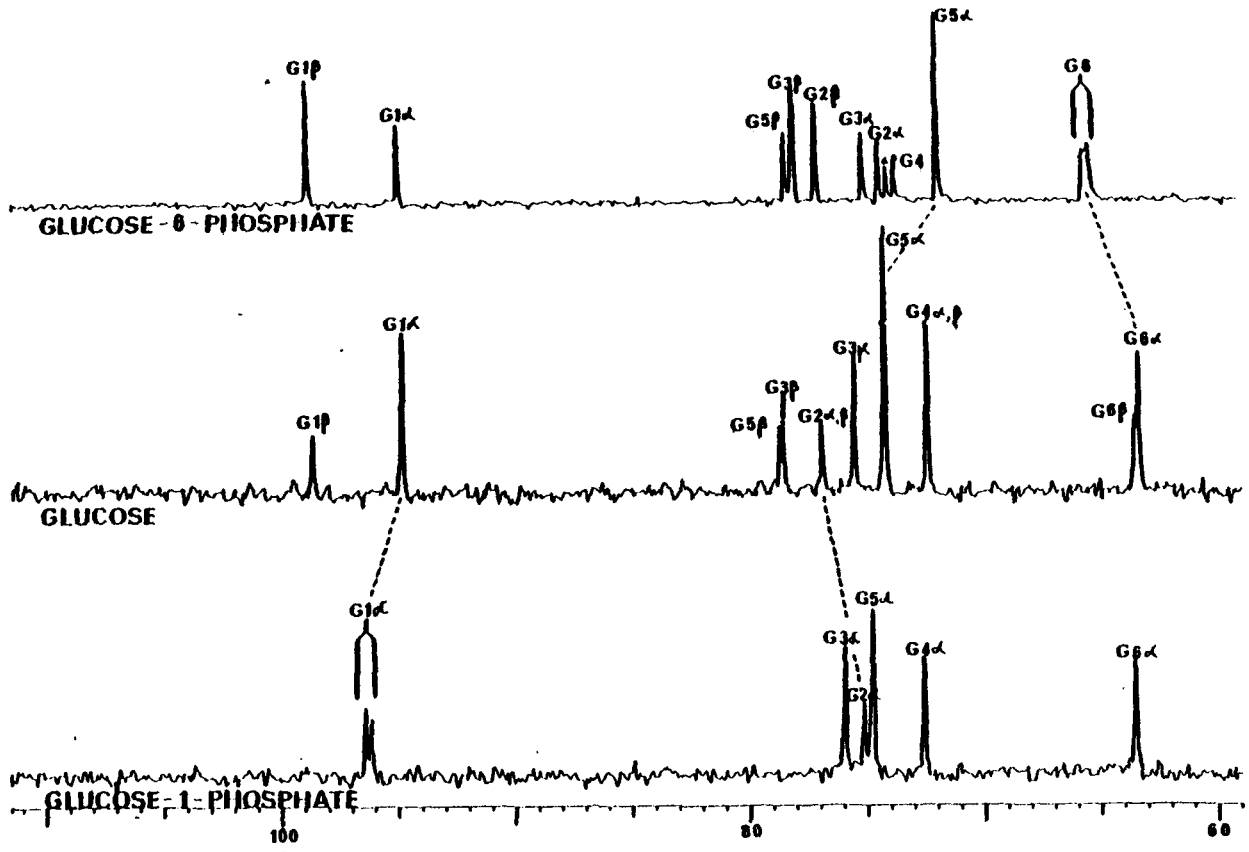

G1β
G1α
G5β
G3β
G2β
G3α
G2α
G4
G5α
G6
GLUCOSE-6-PHOSPHATE
G5α
G1α
G4α,β
G3α
G6α
G1β
G5β
G3β
G2α,β
G6β
GLUCOSE
G1α
G5α
G3α
G4α
G6α
G2α
GLUCOSE-1-PHOSPHATE
100
80
60

TABLE 3.8 *Chemical assignments for ^{13}C-NMR resonance peaks of glucose-1-phosphate glucose-6-phosphate and glucose.*

Glucose-1-phosphate			D-Glucose			Glucose-6-phosphate		
Carbon Assign-ment	Chemical Shift (ppm)	Coupling Constant J (HZ)	Carbon Assign-ment	Chemical Shift (ppm)	Coupling Constant J poc (HZ)	Carbon Assign-ment	Chemical Shift (ppm)	Coupling Constant J (HZ)
C1α	96.348	$^{2}J_{poc}$ = 6.11	C1^{B}	98.705		C1^{B}	98.926	
			C1α	94.859		C1α	95.076	
			C5^{B}	78.715		C5^{B}	78.498	
			C3^{B}	78.553		C3^{B}	78.119	
			C2^{B}	76.927		C2^{B}	77.198	
						C3α	75.194	
C3α	76.006		C3α)	75.573		C2α	74.489	
C2α	75.194		C2α)			C4α	74.164	
C5α	74.814		C5α	94.273		C4^{B}	73.839	
C4α	72.593		C4$\alpha\alpha^{B}$	72.431		C5α	71.997	
C6α	63.600		C6	63.438		C6α^{B}	65.551	$^{2}J_{poc}$ = 3.04

FIGURE 3.8 NMR SPECTRA OF AGROCINOPINE C.

Sucrose and glucose are included for comparison.

Peaks which appear in more than one spectrum are joined by a dotted line. Carbon assignments are indicated above each peak.

Labelling system.

The first letter indicates the molecule from which that carbon originates

G = glucose

and F = fructose.

The number adjacent to that letter is the carbon assignment for that peak, e.g. G2 represents the 2nd carbon in a glucose molecule.

The subscript letter following the numerical value indicates whether the carbon forms part of a sucrose molecule, e.g.

G1s represents the carbon 1 in the glucose portion of sucrose

while G1g represents the carbon 1 in the free glucose molecule.

Conditions of the NMR run.

Solvent:	D_2O
Concentration:	17mg/0.5ml
Reference:	75P
Nucleus:	^{13}C
Frequency:	22.49 MHZ
Offset:	43.2 KHZ
Lock:	2D
Pulse width:	11usec.
Pulse angle:	45
Pulse delay:	2 sec.
Data points:	8k/4k
Window:	10
No. pulses:	35K
Spectral amplitude:	24 x 3db
Spectral width:	5k
Decoupling Mode:	BB
Temperature:	$24^{o}C$.

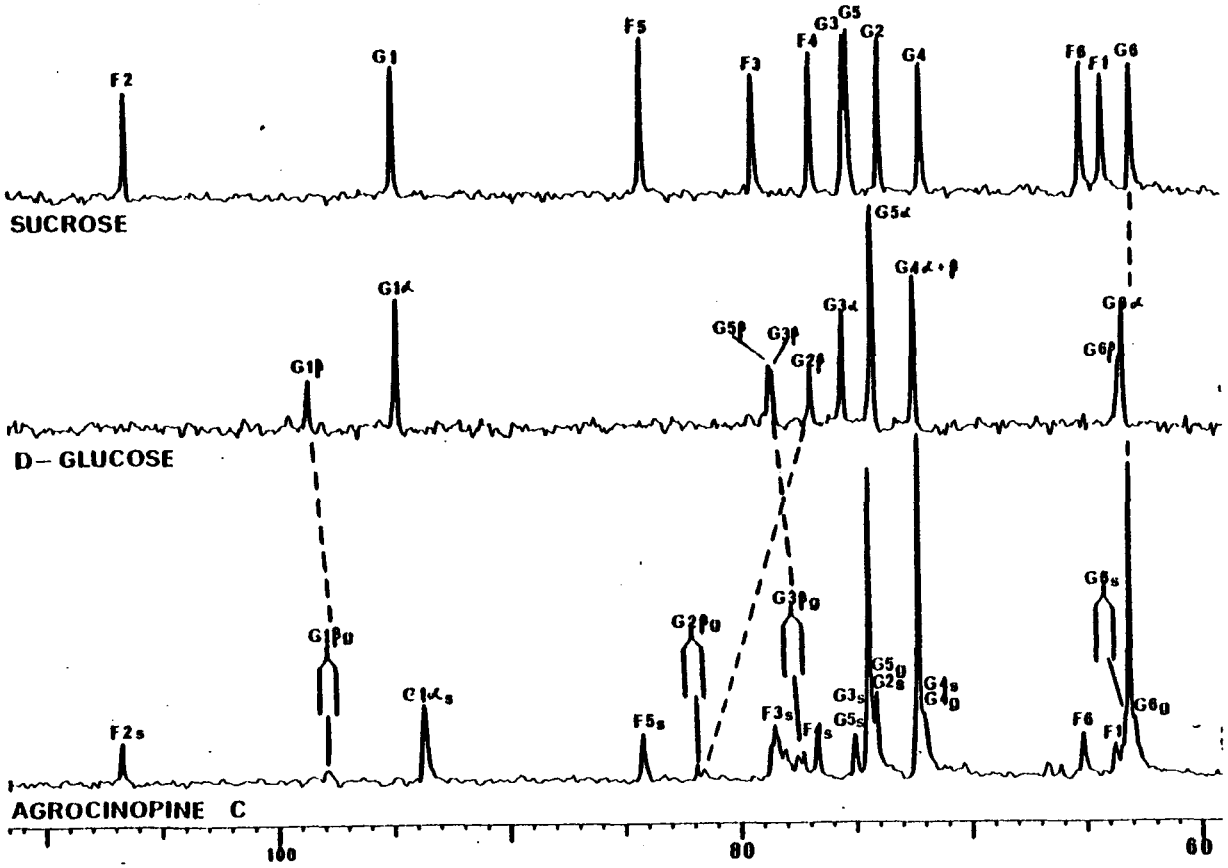

SUCROSE
D– GLUCOSE
AGROCINOPINE C
100
80
60

TABLE 3.9 *Chemical assignments for ^{13}C NMR resonance peaks of Glucose Sucrose and Agrocinopine C*

GLUCOSE			AGROCINOPINE C			SUCROSE		
Carbon Assignment	Chemical Shift (ppm)	Coupling Constant J (HZ)	Carbon Assignment	Chemical Shift (ppm)	Coupling Constant J poc (HZ)	Carbon Assignment	Chemical shift (ppm)	Coupling Constant J (HZ)
$G1^B$	98.705		F2s	106.777	$^3Jpocc = 0.87$	F2	105.965	
G1α	94.859		G1g	98.055		G1α	94.480	
G5B	78.715		G1s	93.776		F5	83.645	
G3B	78.553		F5s	84.295	$^2Jpoc = 1.88$			
G2B	76.927		G2g	81.966		F3	78.878	
G3α)	75.573		F3s	78.607	$^3Jpocc = 6.1$			
G2α)			G3g	77.496		F4	76.386	
G5α	74.273		F4s	76.765		G3α	74.923	
G4$\alpha\alpha$ B	72.431		G3s)	75.1400		G5α	74.706	
G6	63.438		G5s)			G2α	73.406	
			G5g)	74.219		G4α	71.564	
			G2s)			F6	64.630	
			G4s)	72.106		F1	63.763	
			G4g)			G6	62.517	
			F6s	65.226				
			F1s	63.817				
			G6s	63.465	$^2Jpoc = 3.6$			
			G6g	62.9507				

Carbon atom from

G = Glucose

F = Fructose

g indicates free glucose molecule

s indicates that carbon is part of sucrose molecule

FIGURE 3.9 COMPARISON OF THE ^{13}C NMR SPECTRA OF AGROCINOPINE C AND AGROCINOPINE A.

Again corresponding peaks present in both spectrums are joined by a dotted line.

The carbon assignments are given above each peak. The labelling system and conditions of the NMR run are given in Figure 3.8.

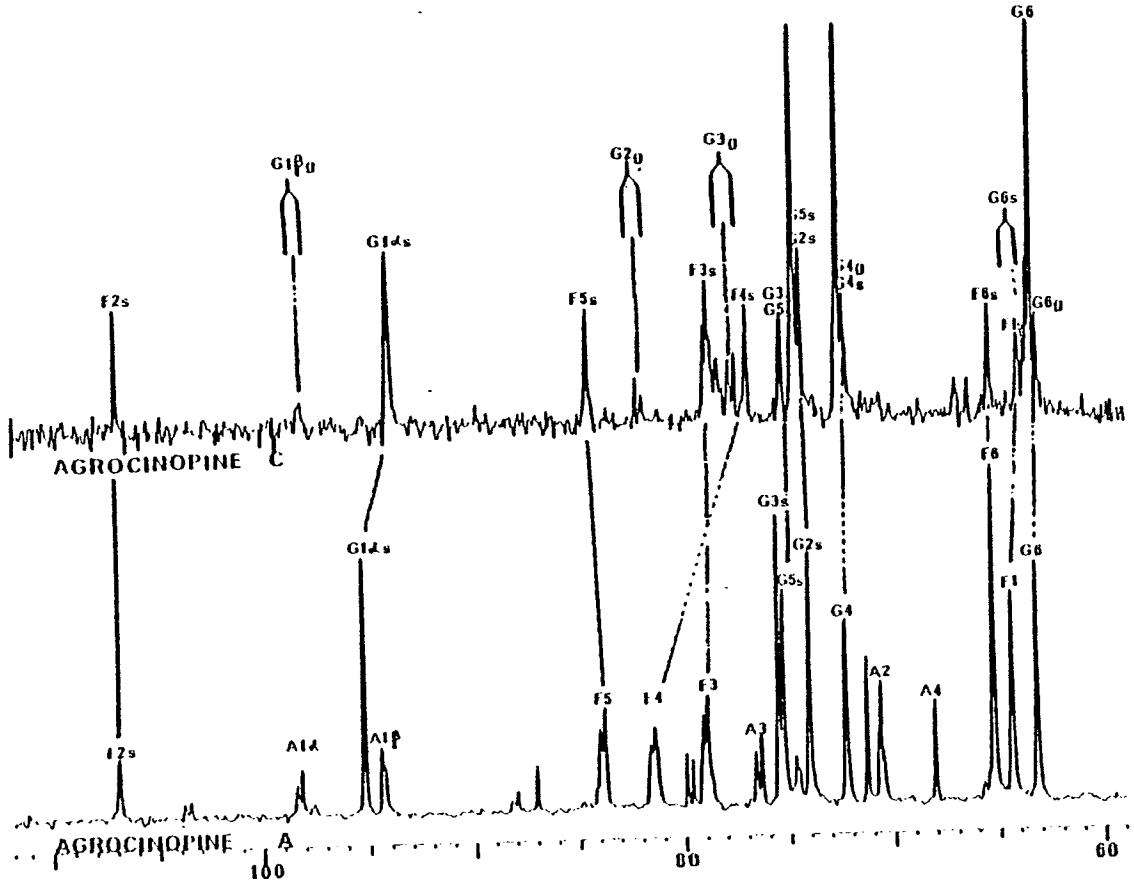

G6
G1β0
G2α0
G3α0
G6s
F2s
G1αs
F5s
F3s
F4s
G3
G5
F6s
G6β0
F6
G3s
G1αs
G2s
G5s
G6
F1
G4
A2
A4
F5
F4
F3
A3
A1α
A1β
f2s
AGROCINOPINE
C
AGROCINOPINE
A
100
80
60

TABLE 3.10 *Chemical assignments for ^{13}C NMR Resonance peaks of Agrocinopine C and Agrocinopine A.*

AGROCINOPINE C			AGRICINOPINE A		
Carbon Assignment	Chemical Shift (ppm)	Coupling Constant J (HZ)	Carbon Assignment	Chemical Shift (ppm)	Coupling Constant J (HZ)
F2s	106.777	$^3J_{pocc}$ = 0.87	F2s	105.1	$^3J_{pocc}$ = 6.1
G1g	98.055		A1α	96.4	
G1s	93.776		G1s	93.4	$^3J_{pocc}$ = 4.9
F5s	84.295	$^2J_{poc}$ = 1.88	A1^B	92.55	$^3J_{pocc}$ = 4.9
G2g	81.966		F5s	82.5	$^2J_{poc}$ = 4.9
F3s	78.607	$^3J_{pocc}$ = 6.1	F4s	79.6	$^2J_{poc}$ = 4.9
G3g	77.496		A2α	77.95	$^3J_{pocc}$ = 4.9
F4s	76.765		F3s	77.2	$^2J_{poc}$ = 4.9
G3s)	75.140		A2^B	74.65	
G5s)			G3s	73.7	
G5g)	74.219		G5s	73.4	$^3J_{pocc}$ = 2.3
G2s)			A3α	72.9	
G4s)	72.106		G2s	72.2	
G4g)			G4s	70.4	
F6s	65.226		A4α	69.4	$^3J_{pocc}$ = 3.7
F1s	63.817		A3^B	68.8	
G6s	63.465	$^2J_{poc}$ = 3.6	A4^B	68.8	
G6g	62.950		A5α	62.4	
			F6s	63.4	
			F1s)	62.6	
			A5^B)		
			G6s	61.4	

TABLE 3.11 *Electrophoretic mobilities of the brief acid Hydrolysis products of Agrocinopine C.*
Formic/acetic acid buffer system pH 1.7.

Compound	RM_{OG}	Staining			
		A	B	C	D
Orange G	1				
xylene cyanol	0.33				
fructose	0	+	+	-	-
Agrocinopine C	0.42	+	+	+	+
Hydrolysis products:					
A	0.57	+	-	+	+
B	1.08	+	-	+	+
C	0	+	+	-	-
D	1.22	+	-	-	+

Stain A = silver nitrate reagent
B = urea phosphoric acid reagent
C = p anisidine
D = phosphate reagent

3.4.1.2 Complete acid hydrolysis.

A 24 hour acid hydrolysis of Agrocinopine D was also performed, again using 1m acetic acid at 110°. Products were separated by H.V.P.E. in an Ammonium Borate buffer system for 1.5 hours at 1500 volts. Reference sugars were included for direct comparison with the hydrolysis products. Hydrolysis products of Agrocinopine D have the same mobilities as the hydrolysis products of glucose-6-phosphate and glucose-1-phosphate (Table 3.12); i.e. two products are produced, one with an electrophoretic mobility corresponding to glucose and one with a mobility identical to that of inorganic phosphate. It should be noted that the sugar allose has an electrophoretic

mobility very close to that of glucose and from this data, its presence in the Agrocinopine D hydrolysis products cannot be discounted.

The glucose oxidase eliminates Allose

TABLE 3.12 *Electrophoric Mobilities of the strong Acid Hydrolysis products of Agrocinopine D.*

~~*Formic/acetic acid buffer system pH 1.7.*~~ Ammonium Borate 9.2.

Compound	RM_{OG}
Orange G	1
xylene cyanol	0.37
fructose	0.79
xylose	0.73
Allose	0.86
Galactose	0.74
Arabinose	0.78
Glucose	0.87
Agrocinopine D	0.71
Hydrolysed Agrocinopine D	0.87
	1.22
Hydrolysed G-6-P	0.87
	1.22
Hydrolysed G-1-P	0.87
	1.22

3.4.1.3 Acid hydrolysis time course.

The rate of acid hydrolysis (1m acetic acid 110°C) of Agrocinopine C was studied by measuring the release of inorganic phosphate from Agrocinopine C as a function of time. The hydrolysis rates of glucose-6-phosphate and glucose-2-phosphate were also examined. The results are presented in Table 3.13. The hydrolysis rate of glucose-2-phosphate is much more rapid than glucose-6-phosphate (Figure 3.10). The results also demon-

TABLE 3.13 *Acid Hydrolysis Time Course.*

Agrocinopine C total P = 0.021umoles.

Time (hours)	Pi (umoles)	% P as Pi
1	0.005	24
1½	0.021	47
2	0.036	59
3	0.040	62
4	0.047	70
5	0.061	82
6	0.079	100
8	0.083	102

Glucose-6-phosphate total P = 0.022umoles.

1	0.005	22
2	0.007	31
3	0.011	50
4	0.014	63
5	0.019	86
5½	0.021	95
6	0.023	104
8	0.022	100

Glucose-2-phosphate total P = 0.032umoles.

½	0.011	34
1	0.023	71
1½	0.027	84
2	0.035	109
2½	0.030	93
8	0.031	96

Linear regression

$Ao = 4.84 \times 10^{-3} \pm 9.5 \times 10^{-4}$

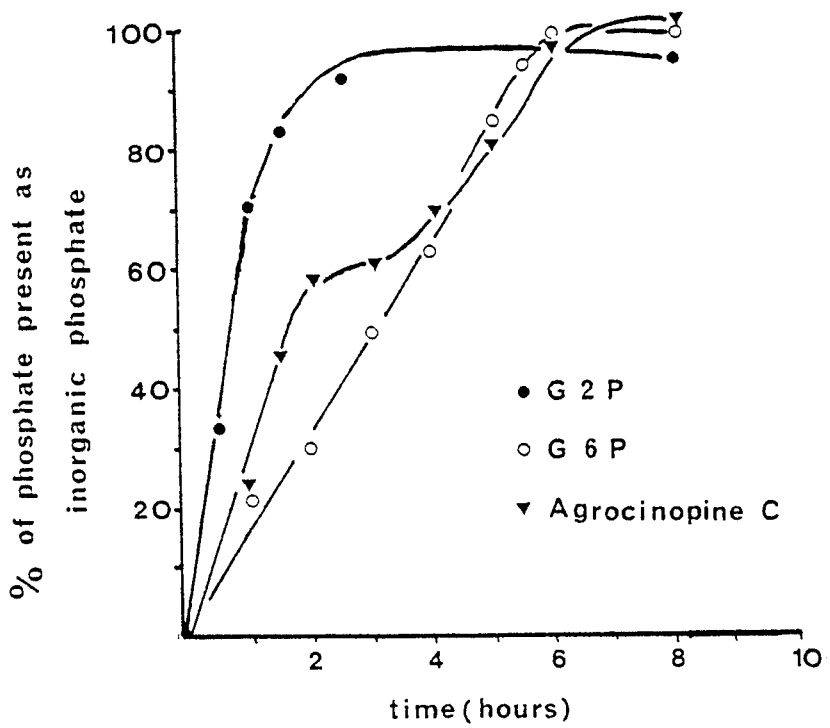
$A_1 = 2.07 \times 10^{-1} \pm 7.4 \times 10^{-4}$

$r^2 = 0.995$

FIGURE 3.10 ACID HYDROLYSIS TIME COURSE.

The rate of acid hydrolysis was measured by measuring the release of inorganic phosphate (vertical axis) as a function of time (horizontal axis).

ACID HYDROLYSIS TIME COURSE



strate that the acid hydrolysis of Agrocinopine C occurs in two steps. The first involves a rapid release of inorganic phosphate, followed by a much slower breakdown rate.

3.4.2 Ammonolysis.

Agrocinopine C was subjected to Ammonolysis in 1.5m Ammonium Solution for 40 minutes at 110°C. The products were examined by H.V.P.E. in the formic/acetic buffer system pH 1.7. The electrophoretic mobilities can be seen in Table 3.14. Ammonolysis yielded three products. The non migrating product (A) was the only spot which stained with the urea phosphoric reagent, whilst the second product (B) stained only with the phosphate reagent and has similar mobility to inorganic phosphate.

TABLE 3.14 *Relative Mobilities for the Ammonolysis Products of Agrocinopine C in formic/acetic acid buffer system pH1.7.*

Compound	RM_{OG}	Staining A	B	C
Orange G	1			
xylene cyanol	0.32			
Sucrose	0	+	+	-
Ammonolysis products:				
A	0	+	+	-
B	0.53	-	-	+
C	0.61	+	-	+

Stains
A = silver nitrate
B = urea phosphoric reagent
C = phosphate reagent

3.4.2.1 Properties of Ammonolysis Product C.

Ammonolysis Product C which reacted positively with both the silver nitrate and the phosphate reagent was eluted from a formic/acetic acid buffer electrophoretogram and examined under a number of different treatments.

3.4.2.2 Ammonium borate electrophoresis.

The electrophoretic mobilities of ammonolysis product C on H.V.P.E. in the ammonium borate buffer pH 9.2 are listed in Table 3.15. In this system product C was resolved into 4 distinct components all of which stained with the phosphate reagent, the silver nitrate reagent and p anisidine.

TABLE 3.15 *Relative Mobilities of Ammonolysis product C on H.V.P.E. in an Ammonium Borate System pH 9.2.*

Compound	RM_{OG}	Staining			
		A	B	C	D
Orange G	1				
xylene cyanol	0.37				
Glucose	0.91	+	+	-	-
Sucrose	0.09	+	+	+	-
Ammonolysis Product C	0.81	+	+	-	+
	0.97	+	+	-	+
	1.11	+	+	-	+
	1.27	+	+	-	+

A = silver nitrate
B = p anisidine
C = phosphate reagent
D = urea-phosphoric reagent

3.4.2.3 Treatment with alkaline phosphatase.

Ammonolysis product C was also treated with alkaline phosphatase (E.C. 3.1.3.1) 32°C 2 hours. The enzyme was removed by precipitation in 70% ethanol and the products examined by H.V.P.E. in the ammonium borate buffer system at pH 9.2. Again the appropriate sugar standards were included on the electrophoretogram. A control sample of the enzyme (alkaline phosphatase) was precipitated in 70% ethanol and also run on this electrophoretogram, in order to check that it did not interfere with the results. It can be seen (Table 3.16) that the alkaline phosphatase treatment of the ammonolysis Product C yields inorganic phosphate and one other product with a

mobility identical to that of glucose.

TABLE 3.16 *Relative Mobilities in the Ammonium Borate buffer system pH 9.2 of the Ammonolysis Products after treatment with Alkaline Phosphatase.*

Compound	RM_{OG}
Orange G	1
Xylene cyanol	0.29
Agrocinopine C	0.42
Enzyme A.P.	0.32
Enzyme	-
Glucose	0.32

3.4.2.4 Treatment with Glucose Oxidase.

Ammonolysis product C was treated with alkaline phosphatase to cleave the phosphate monoesters. The enzyme was again removed by precipitation in 70% ethanol and the products of this reaction treated with D-glucose oxidase (E.C. 1.1.3.4) 1 hour room temp. Table 3.17 shows the electrophoretic mobilities of the products of this reaction sequence.

TABLE 3.17 *Relative mobilities in Ammonium Borate buffer pH 9.2 of the Ammonolysis products after treatment with alkaline phosphatase and treatment with glucose oxidase.*

Compound	RM_{OG}
Orange G	1
Xylene cyanol	0.29
Ammonolysis P. treated $\bar{c}$ Phosphatase	0.83
Ammonolysis P. treated $\bar{c}$ Phosphatase & Glucose oxidase	1.07
Glucose	0.83
Glucose treated $\bar{c}$ Glucose oxidase	1.07
Gluconate	1.07
Glucitol	0.93

3.4.3 Reduction of Agrocinopine C.

Agrocinopine C was reduced with $NaBH_4$ and the products examined on H.V.P.E. in the formic/acetic buffer system pH 1.7. Reduced Agrocinopine C had a higher mobility than Agrocinopine C and no longer stained with the p anisidine stain (Table 3.18).

TABLE 3.18 *Reduction of Agrocinopine C; Electrophoretic Mobilities in the formic/acetic acid system pH 1.7.*

Compound	RM_{OG}
Orange G	1
X C	0.33
Agrocinopine C	0.48
Reduced Agrocinopine C	0.55

3.4.3.1 Ammonolysis of Reduced Agrocinopine C.

Reduced Agrocinopine C was subjected to Ammonolysis, followed by treatment with alkaline phosphatase. These reactions were examined by H.V.P.E. in both the ammonium borate buffer system pH 9.2, (Table 3.19) and the formic/acetic lanthanum complexing buffer system pH 1.7 (Table 3.20). The final product was indistinguishable from glucitol in both buffer systems.

TABLE 3.19 *Ammonolysis of Reduced Agroncinopine C: Electrophoretic Mobilities in H.V.P.E. in ammonium borate buffer pH 9.2.*

Compound	RM_{OG}
Ammonolysis product cleaved with Alkaline Phosphatase	0.78
Reduced Ammonolysis product cleaved with Alkaline Phosphatase	0.94
Glucose	0.78
Glucitol	0.94

TABLE 3.20 *Ammonolysis of Reduced Agroncinopine C: Electrophoretic Mobilities in H.V.P.E. in Lanthanum complexing buffer pH 1.7.*

Compound	RM_{OG}
Xylene cyanol	0.12
Orange G	1
Glycerol	-0.15
Sucrose	0.0
Ribitol	-0.17
Glucose	0
Glucitol	-0.71
Reduced Amm.P.Alkaline Phosphatase treated	-0.71
Reduced Amm. Product	-0.23

Figure 3.11 shows the electrophoretogram from which these mobilities were taken.

FIGURE 3.11 REDUCTION AND AMMONOLYSIS OF AGROCINOPINE C.

Buffer system: Ammonium Borate
Stain: Alkaline silver nitrate reagent.
Conditions: 1500v 45 minutes

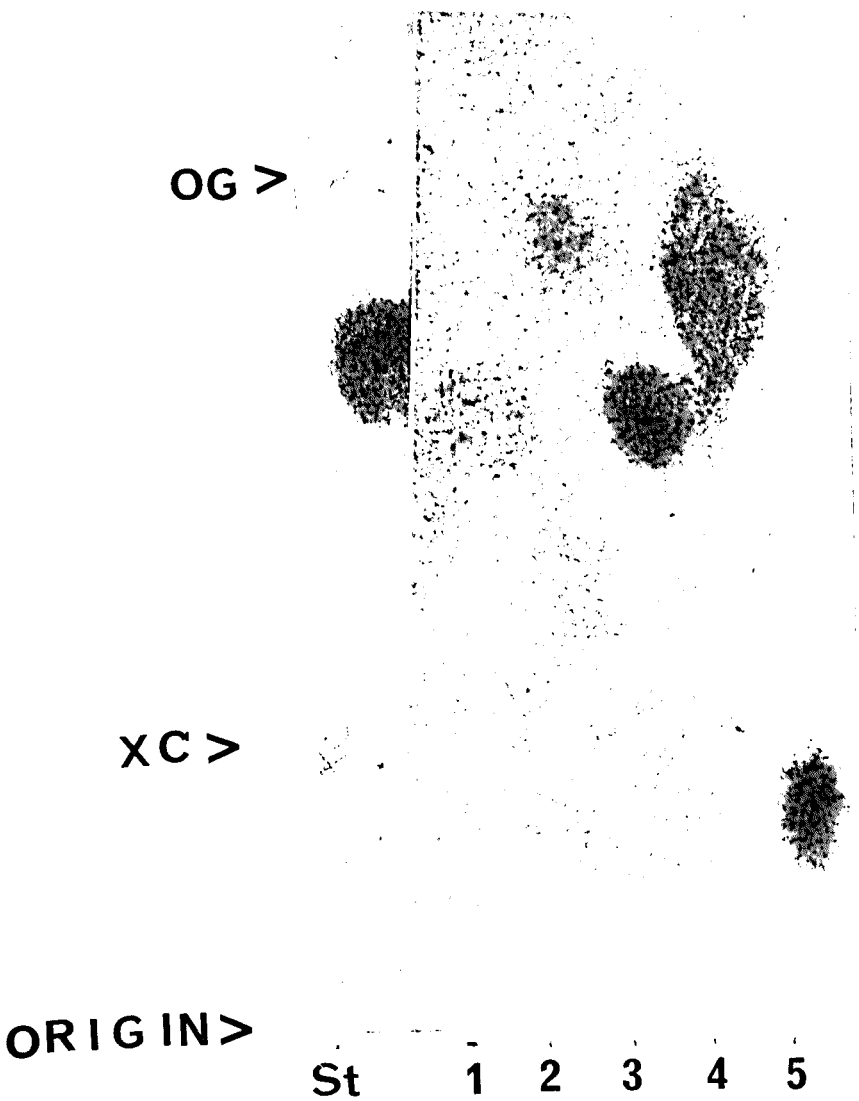
Reduced Agrocinopine C was subjected to the following treatments.

(1) Ammonolysis (1.5m NH_4OH 110°C 40 min.)
(2) Alkaline Phosphatase digestion E.C. 3131.

The product of this reaction sequence is shown in lane 2.

Similar treatment of the unreduced Agrocinopine C yielded the products in lane 1.

Lane 3 contains a D-glucose standard and lane 4 a glucitol standard.


OG >
XC >
ORIGIN>
St
1
2
3
4
5

3.4.4 Quantitative Measurement of the Individual Components in Agrocinopine C.

3.4.4.1 Total Phosphorous.

The total phosphorous content of a standard sample of Agrocinopine C was measured using Bartletts (1959) method of phosphate determination. The results can be seen in Table 3.21.

TABLE 3.21

Compound	Number of umoles	Absorbance
Standard K_2HPO_4	0.020	0.105
	0.050	0.275
	0.100	0.320
	0.150	0.708
0.094mg Agrocinopine C	0.170*	0.776

Linear regression

$A_o = 5.13 \times 10^{-3} \pm 2.2 \times 10^{-2}$

$A_1 = 2.12 \times 10^{-1} \pm 2.09 \times 10^{-2}$

$r^2 = 0.89$

* umoles calculated from the absorbance values using the linear regression.

3.4.4.2 Total Sugar.

Total sugar was measured using the phenol-sulphuric acid method (Dubois et al 1956). The results can be seen in Table 3.22.

FIGURE 3.12 AN ELECTROPHORETOGRAM OF THE GLUCOSE PHOSPHATES.

Buffer system: 0.2m Ammonium borate
Stain: Phosphate reagent
Conditions: 1500v 40 mintues.

Lane 1 = G-1-P
2 = G-2-P
3 = G-3-P
4 = G-4-P
5 = G-6-P

St = Standard solution containing Orange G (OG) and xylene cyanol (XC)

The origin is marked by a dotted line.

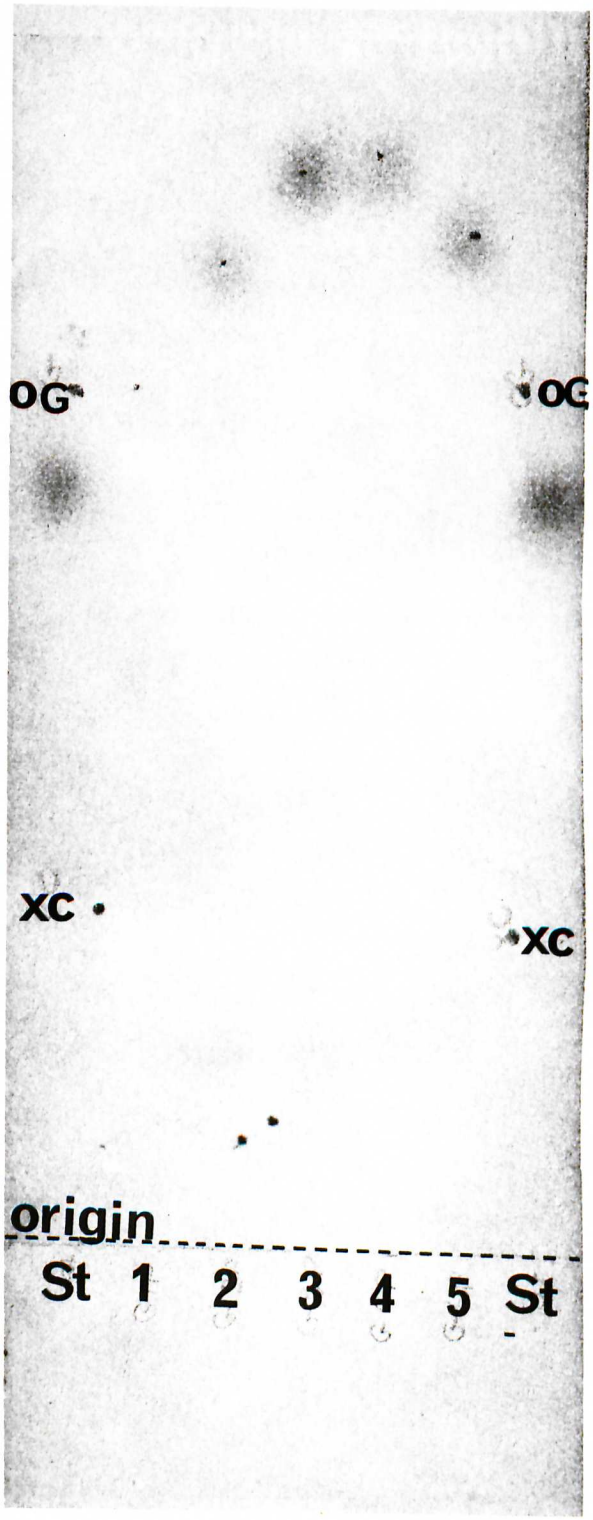

OG
OG
XC
XC
origin
St 1 2 3 4 5 St

TABLE 3.22

Compound	Number of umoles	Absorbance
Standard	0.112	0.186
D-Glucose	0.224	0.387
	0.336	0.596
	0.448	0.77
0.023mg Agrocinopine C	0.138*	0.236
4.7ug Agrocinopine C	0.026*	0.038

Linear regression

$A_o = 5.31 \times 10^{-3} \pm 5.12 \times 10^{-3}$

$A_1 = 5.64 \times 10^{-1} \pm 6.1 \times 10^{-3}$

$r^2 = 0.99$

* concentration calculated from the absorbance values using the linear regression.

3.4.4.3 Total D-glucose content.

The glucose content of a purified Agrocinopine C sample was measured using a glucose oxidase assay (Bergmeyer 1971). The results can be seen in Table 3.23.

3.4.4.4 Comparison of phosphorous, sugar and glucose content.

From the total phosphorous, total sugar and glucose measurements, a ratio of phosphorous : sugar : glucose can be calculated.

Agrocinopine C contains:

0.64umoles of phosphorous/ug

1.96umoles of sugar/ug

and 1.20umoles of D-glucose/ug.

Thus the ratio of P : Sugar : D-glucose

is 1 : 3.06 : 1.87

corresponding to integer values of 1 : 3 : 2.

TABLE 3.23

Compound	Number of umoles	Absorbance
Standards D-glucose	0.0028	0.051
	0.0056	0.103
	0.0086	0.185
	0.0112	0.242
	0.0140	0.307
	0.0168	0.382
	0.028	0.598
4.7ug Agrocinopine C	0.0155*	0.377
9.4ug Agrocinopine C	0.0250*	0.547

Linear regression

$A_o = 3.86 \times 10^{-4} \pm 5.4 \times 10^{-4}$

$A_1 = 4.51 \times 10^{-2} \pm 3.8 \times 10^{-4}$

$r^2 = 0.996$

* Number of umoles calculated from the absorbance values using the linear regression.

3.4.5 Glucose Phosphates.

3.4.5.1 Electrophoretic mobilities.

Mobilities relative to Orange G in H.V.P.E. of the various glucose phosphates have been measured in several different buffer systems and the results are presented in Table 3.24. Figure 3.12 shows their mobility in the 0.8m Ammonium Borate buffer pH 9.2. Glucose-3-phosphate and

glucose-4-phosphate are barely separable in any of the systems used, while glucose-1-phosphate, glucose-2-phosphate and glucose-6-phosphate are clearly resolvable in most buffer systems.

TABLE 3.24 *Relative Mobilities of the Glucose Phosphates.*

Compound	RM_{OG}					
	A	B	C	D	E	F
Orange G	1	1	1	1	1	1
xylene cyanol	0.36	0.39	0.39	0.12	0.53	0.33
G-1-P	1.04	1	1.05	-0.52	1.24	0.66
G-2-P	1.10	1.13	1.16	-0.58	1.36	0.66
G-3-P	1.23	1.24	1.28	-0.54	1.33	0.66
G-4-P	1.23	1.25	1.28	-0.54	1.33	0.66
G-6-P	1.15	1.17	1.23	-0.54	1.16	0.66

A 0.2m ammonium borate pH 9.2
B 0.8m ammonium borate pH 9.2
C 1.2m ammonium borate pH 9.2
D lanthanum/formic/acetic pH 1.7
E citrate buffer pH 6.5
F formic acetic acid pH 1.7

The sugar phosphates were also examined by paper chromatography in the propanol/water/ammonium (7 : 2 :1) solvent system. The mobilities relative to the solvent front are presented in Table 3.25.

TABLE 3.25 *Glucose Phosphates: Mobilities relative to the Solvent front in Paper chromatography solvent system = NH_4OH : Propanol : H_2O (2 : 7 : 1).*

Mobilities relative to solvent front:

Compound	Rf
Xylene cyanol	0.86
Orange G.	0.72
Fructose	0.65
G-1-P	0.18
G-2-P	0.22
G-3-P	0.23
G-4-P	0.22
G-6-P	0.14
Glucose	0.625
Agrocinopine C	0.48

3.4.5.2 Electrophoretic mobilities of the reduced glucose phosphates.

The electrophoretic mobilities of the reduced glucose phosphates were also examined on H.V.P.E. in the 0.2m ammonium borate buffer system. The results are shown in Table 3.26.

TABLE 3.26 *The Reduced Glucose Phosphates: Mobilities in the 0.2m ammonium borate buffer system.*

Compound	RM_{OG}
Orange G	1
xylene cyanol	0.36
Reduced G-1-P	1.06
Reduced G-2-P	1.22
Reduced G-3-P	1.29
Reduced G-4-P	1.23
Reduced G-6-P	1.23

3.4.5.3 Comparison of the glucose phosphates with the ammonolysis Products C.

The electrophoretic mobilities of the ammonolysis Products C and the glucose phosphates were compared on H.V.P.E. in an ammonium borate buffer system at 15v for 1.5 hours (Table 3.27). Ammonolysis Products C have the same mobility as G-1-P, G-2-P and G-3-P.

TABLE 3.27 *Comparison of Relative Mobilities of the glucose phosphates with those of the Ammonolysis Products C on 0.2m ammonium borate buffer system pH 9.2.*

Compound	RM_{OG}
Orange G	1
xylene cyanol	0.36
G-1-P	1.04
G-2-P	1.10
G-3-P	1.23
G-4-P	1.23
G-6-P	1.15
Ammonolysis Product C	0.87
	1.00
	1.11
	1.25

The Ammonolysis Products C were also reduced and compared with the reduced glucose phosphates on H.V.P.E. in an ammonium borate buffer pH 9.2. (Table 3.28).

TABLE 3.28 *Comparison of the Relative Mobilities in an ammonium borate system pH 9.2 of the reduced glucose phosphates with the reduced Ammonolysis products.*

Compound	RM_{OG}
Orange G	1
xylene cyanol	0.36
Reduced G-1-P	1.06
Reduced G-2-P	1.22
Reduced G-3-P	1.29
Reduced G-4-P	1.23
Reduced G-6-P	1.23
Reduced Ammonolysis Products	1.06
	1.22
	1.27

3.4.5.4 Bioassay.

Glucose-2-phosphate and glucose-6-phosphate were tested for biological activity in the standard plate bioassay. Figure 3.13 shows their effect on the sensitivity to Agrocin 84 in the normally insensitive strain K478 and Figure 3.14 illustrates their effect on sensitivity to Agrocin 84 in the normally sensitive strain K476.

FIGURE 3.13 BIOASSAY OF GLUCOSE-2-PHOSPHATE AND GLUCOSE-6-PHOSPHATE USING THE INSENSITIVE STRAIN K478 AS THE INDICATOR STRAINS.

A = G-2-P
B = G-6-P
C = Agrocinopine C

Plate 1 shows that G-2-P and G-6-P are biologically inactive on strain K478, i.e. they do not induce sensitivity to Agrocin 84.

Plate 2 is the same bioassay, but including Agrocinopine C. The biological activity of Agrocinopine C can be clearly seen, while G-2-P and G-6-P remain inactive.

Plate 3 is a control plate showing that the glucose phosphates have no effect in the absence of Agrocin 84.

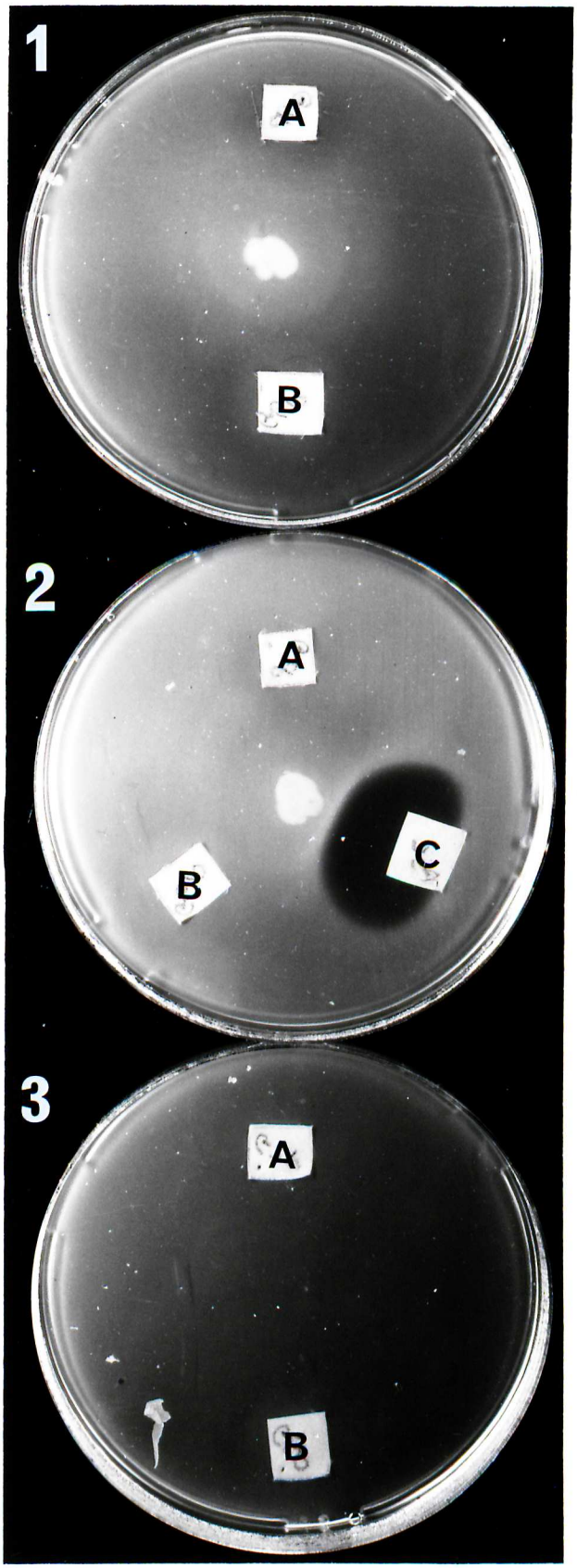

1
A
B
2
A
B
C
3
A
B

FIGURE 3.14 BIOASSAY OF GLUCOSE-2-PHOSPHATE AND GLUCOSE-6-PHOSPHATE USING K476 AS THE INDICATOR STRAIN.

A = G-2-P
B = G-6-P
C = Agrocinopine C

Plate 1 is a control plate containing no Agrocin 84. G-2-P and G-6-P have no activity in the absence of Agrocin 84.

Plate 2 shows the biological activity of G-2-P, G-6-P and Agrocinopine C. It can be seen that G-2-P and Agrocinopine C counteract the toxic effect of Agrocin 84 on the sensitive strain K476 This effect is very slight. The arrows point to the faint indentation in the inhibition zone.

Plate 3 shows the same bioassay of G-2-P and G-6-P. Again the arrow points to the slight indentation in the inhibition zone.

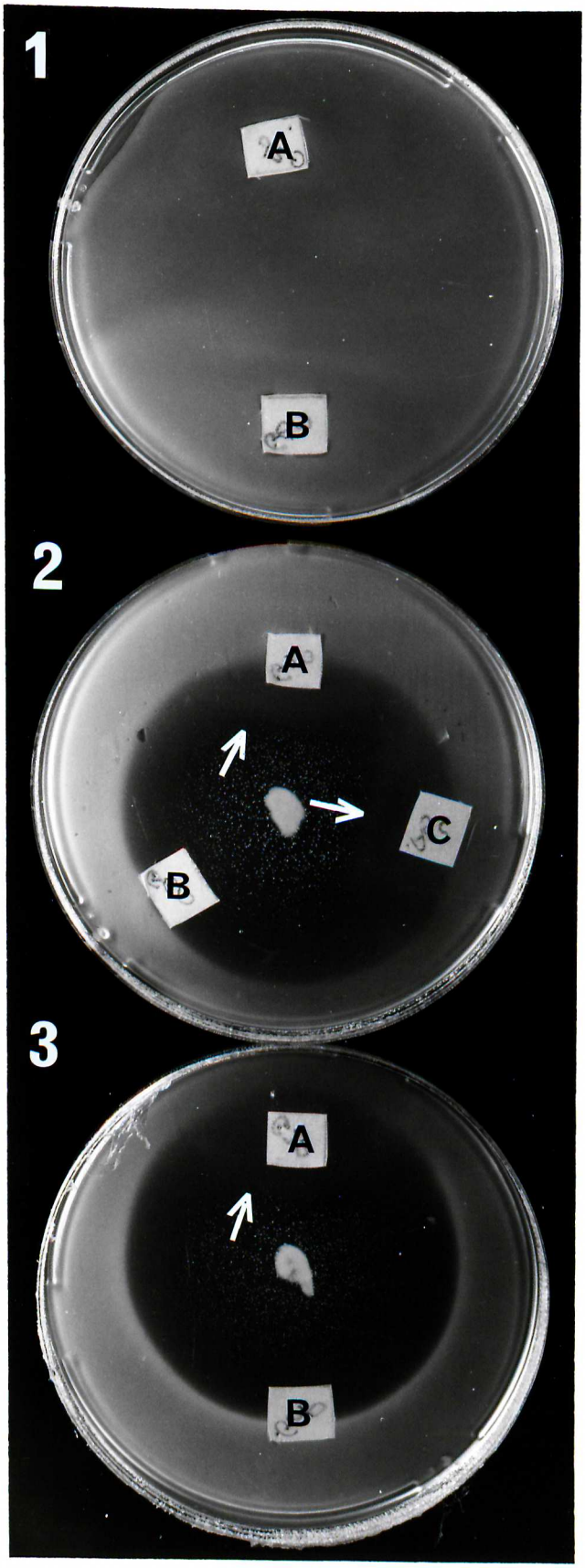

1
A
B
2
A
C
B
3
A
B

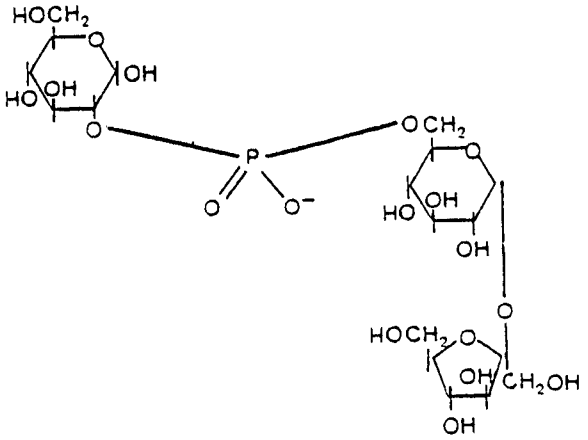
FIGURE 4.1 THE PROPOSED STRUCTURES OF AGROCINOPINE C AND D.

Agrocinopine C consists of a sucrose molecule, linked through the hydroxyl group on the 6th carbon of the glucose moiety via a phosphodiester bond to a free glucose molecule linked through the second carbon.

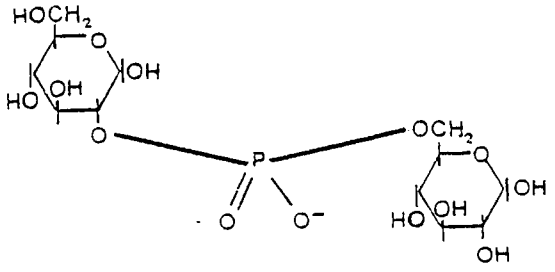
Agrocinopine D is closely related, the only difference being the loss of a fructose molecule.

PROPOSED STRUCTURES-

AGROCINOPINE C



AGROCINOPINE D



4. DISCUSSION

4.1 FORWARD

The proposed structures of Agrocinopine C and D are shown in Figure 4.1. Agrocinopine C consists of a glucose-2-phosphate linked via a phosphodiester bond to the hydroxyl group of the carbon 6 of the glucose moiety of a sucrose molecule. It can be seen that Agrocinopine D is closely related to Agrocinopine C, the only difference being the loss of the fructose molecule. In this section I shall attempt to explain how the experimental evidence presented, leads to the proposed structures of Agrocinopine C and D.

4.2 PURIFICATION

The structural elucidation of any naturally occurring product begins with its isolation and purification. Purity of the Agrocinopine C is essential if meaningful information is to be derived from the physical and degradative studies.

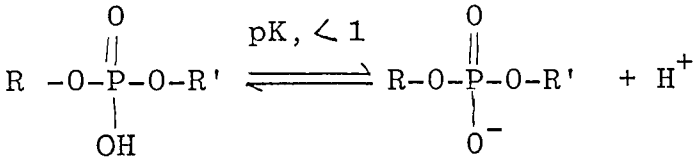
Agrocinopine C was known (Ellis & Murphy 1981) to be anionic by H.V.P.E., and thus an anion exchange column in the acetate form was chosen as the first step in the purification procedure. In this way most of the cationic and neutral components in the crude extract, were removed. Purification at pH 9.2 via a second anion exchange column in the bicarbonate form served to resolve phosphate monoesters from diesters as shown in the mobility profile (Figure 3.4).

Agrocinopine C was also found to complex strongly with Lanthanum in H.V.P.E., and a cation exchange column in the Lanthanum form was consequently chosen as the next step in the purification. Unfortunately in the column situation Agrocinopine C did not complex with Lanthanum and was eluted with the solute front, appearing in fractions 2, 3 and 4. Nevertheless, residual cationic material was retarded and further purification was achieved.

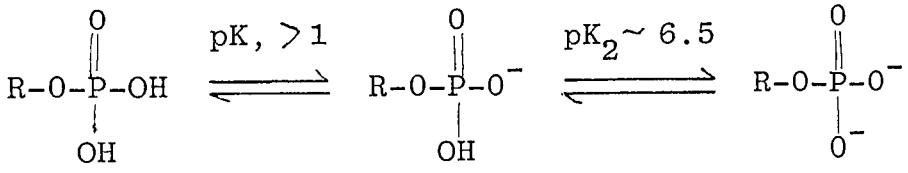
At this stage only one impurity (non phosphorylated) could be detected in the Agrocinopine C sample. This impurity could be detected only in the formic/acetic acid buffer system pH 1.7 and only with the silver nitrate detection reagent. Much of the degradative work was carried out at this stage of the purification, the reactions being followed by staining primarily with the phosphate reagent but also with the urea phosphoric and p anisidine reagents. Since the impurity present did not react with any of these stains it did not interfere with the interpretation of the degradative results. Agrocinopine C was further purified for the physico-chemical studies by H.V.P.E. The impurity present was unresolved from Agrocinopine C at any pH except pH 1.7. H.V.P.E. was chosen in preference to an anion exchange column, to reduce the time that Agrocinopine C was subjected to acid conditions. Agrocinopine C was eluted with dilute ammonia solution, again to reduce breakdown under acid conditions.

4.3 ELECTROPHORETIC pH MOBILITY PROFILE : CHARGE CHARACTERISTICS

Much useful information may be inferred from the pH-mobility profiles (Figure 3.4). Agrocinopines C and D have flat profiles indicating that these molecules are completely ionised in the observed pH range as in the case of bis p-nitrophenyl phosphate (Tate 1968).



Phosphomonoesters on the other hand undergo two dissociation steps;



as illustrated by the pH mobility profile (Figure 3.4) for ribose-5-phosphate.

4.4 IDENTIFICATION OF THE INDIVIDUAL COMPONENTS OF AGROCINOPINE C

Qualitative work done by Ellis and Murphy (1981) suggested that Agrocinopine C contained a glucose molecule, a fructose molecule and a phosphate group. The next step in the structural characterisation of Agrocinopine C and D was the confirmation of these results.

4.4.1 Acid Hydrolysis.

Weak acid hydrolysis of Agrocinopine C yielded Agrocinopine D and fructose (Table 3.11) This result was important because Agrocinopine C reacts positively with the urea phosphoric reagent for ketoses, (Table 3.6) whilst Agrocinopine D does not. The clear inference from these observations is that there is a very acid labile ketose (fructose) moiety in Agrocinopine C. This data contrasts strongly with Agrocinopine A which has a stable fructose moiety (Agrocinopine B is ketose-positive), but exhibits a very acid labile glucose moiety. Further acid hydrolysis of Agrocinopine D yielded only two components, which were indistinguishable from glucose and inorganic phosphate (Table 3.12).

4.4.2 Ammonolysis.

Ammonolysis of Agrocinopine C yields a sucrose molecule and a mixture of four sugar phosphates (Table 3.15). These sugar phosphates were treated with alkaline phosphatase to yield a product which was indistinguishable from glucose (Table 3.16). Some monosaccharides are barely resolvable by H.V.P.E. in borate buffers, for example glucose has an RM_{OG} = 0.87 while allose has an RM_{OG} = 0.86 in the ammonium borate buffer pH 9.2. Thus at this stage the free sugar in Agrocinopine C could not be identified as glucose with any certainty. Treatment

of this product with D-glucose oxidase led to the complete disappearance of the glucose and the appearance of a new product with a mobility identical to that of Gluconate (Table 3.17). D-glucose oxidase, is highly specific for D-glucose, thus this reaction sequence provided concrete evidence that the non-sucrose sugar in Agrocinopine C was D-glucose.

4.4.3 Reduction of Agrocinopine C.

Further evidence for the presence of glucose as the non-sucrose component of Agrocinopine C came from borohydride reduction studies. Agrocinopine C is easily reduced with $NaBH_4$ and subsequent ammonolysis and alkaline phosphate cleavage yielded a product which was indistinguishable from glucitol (the reduced counterpart of glucose) in both buffer systems tried (Table 3.19 and 3.20, Figure 3.11). The Lanthanum complexing buffer system is particularly useful for resolving the Polyols.

Thus from the degradative studies it became clear that Agrocinopine C consisted of a sucrose molecule joined by a phosphodiester linkage to a D-glucose molecule, and that Agrocinopine D had simply lost the fructose portion of the sucrose moiety in Agrocinopine C.

Quantitative work confirmed this hypothesis giving a phosphorous : total sugar : D-glucose ratio of 1 : 3 : 2 - that is, for every phosphate molecule in Agrocinopine C there are 2 D-glucose molecules and a fructose molecule.

4.5 INFRA RED SPECTRA

Comparison of the spectra for sucrose, glucose-2-phosphate and Agrocinopine C (Figure 3.5) show that they are closely related molecules. The spectra for Agrocinopine A and C also show similarities (Figure 3.6).

4.6 LOCATION OF THE PHOSPHATE LINKAGES

The next step in the structural elucidation of Agrocinopine C was the location of the phosphate linkages. It was decided that the best way to identify the phosphate linkages in Agrocinopine C was by comparison of the four sugar phosphates obtained by ammonolysis (which had been identified as glucose phosphates) with the various glucose phosphates. As already indicated G-1-P and G-6-P are readily available "off the shelf" but it was necessary to synthesise G-2-P (Figure 2.2), G-3-P (Figure 2.4) and G-4-P (Figure 2.3).

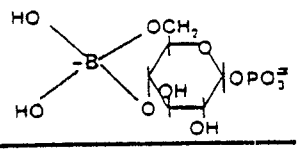
4.6.1 Electrophoretic Mobilities of the Glucose Phosphates.

The mobility of the glucose phosphate standards relative to O.G. was examined under a number of

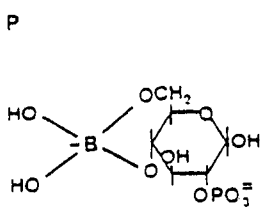
FIGURE 4.2 THE BORATE COMPLEXING BEHAVIOUR OF THE GLUCOSE PHOSPHATES.

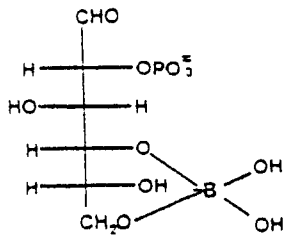
The borate complexing behaviour of the glucose phosphates is influenced by both the position of the phosphate and the concentration of the borate.

G-1-P

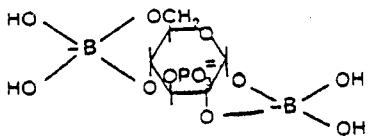


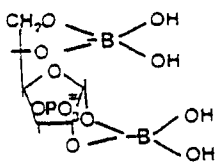
G-2-P

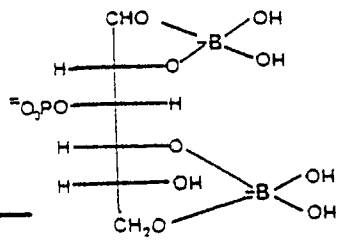




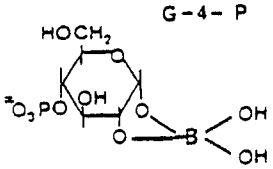
G-3-P

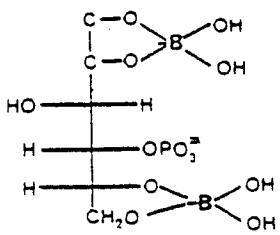


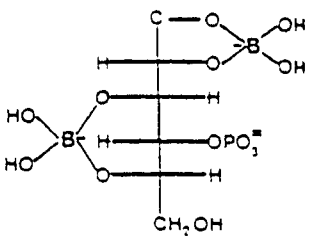




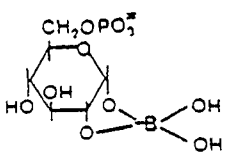
G-4-P

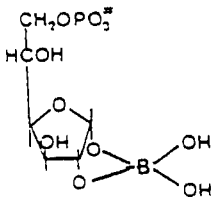






G-6-P





different buffer systems, in order to obtain the best conditions for their resolution. The glucose phosphates all have exactly the same charge characteristics and size, and thus it was thought that the borate complexing buffer system would be the most appropriate for their separation. Several different concentrations of borate (0.2m - 1.2m) were tried on the assumption that both the position of the phosphate group and the concentration of borate would influence borate complexing ability. The expected borate complexing behaviour of the glucose phosphates can be seen in Figure 4.2. Glucose-1-phosphate would be expected to form only one borate complex, bridging the hydroxyl groups on carbon 4 and 6, while glucose-3-phosphate would be expected to form 3 borate complexes all with an overall charge of -4. These complexes are based on the glucose-3-phosphate in the furanose ring form, the pyranose ring form and the linear form. Good separations of glucose-1-phosphate, glucose-2-phosphate and glucose-6-phosphate were obtained in all borate complexing systems. However glucose-3-phosphate and glucose-4-phosphate proved very difficult to separate (Table 3.24).

A Lanthanum complexing buffer system was also tried to obtain better resolution between G-3-P and G-4-P without success. The final buffer system tried was a sodium citrate buffer at pH 6.5. The glucose phosphates have Pka values 6.5 and thus separations would be expected to be maximised at this pH but

again no satisfactory resolution was obtained.

Further attempts to distinguish the glucose phosphates in H.V.P.E. by reduction of the phosphates to the corresponding glucitol phosphates were also made (Table 3.26). Because of its anomeric phosphate substituent, glucose-1-phosphate is not reduced and consequently its mobility and its p anisidine reaction is not altered. Reduced G-2-P has a much higher mobility than its unreduced counterpart as does reduced G-6-P. Reduced G-4-P moves more slowly than unreduced G-4-P, while the mobility of reduced G-3-P is similar to that of the unreduced compound, and hence the glucose 3 and 4 phosphates could be distinguished by this means.

4.6.2 Comparison of the glucose phosphates with the ammonolysis products from Agrocinopine C degradation.

Comparison of the glucose phosphates with the sugar phosphates obtained after ammonolysis of Agrocinopine C (Table 3.27) and of the reduced glucose phosphates with the reduced sugar phosphates from ammonolysis (Table 3.28) led to the conclusion that the four sugar phosphates obtained after ammonolysis of Agrocinopine C consisted of a mixture of a cyclic glucose phosphate, G-1-P, G-2-P and G-3-P. The cyclic glucose phosphate was identified by the fact that the cyclic sugar phosphates behave as phosphodiesters in H.V.P.E., and their electro-

phoretic mobility remains the same at a number of different pH's.

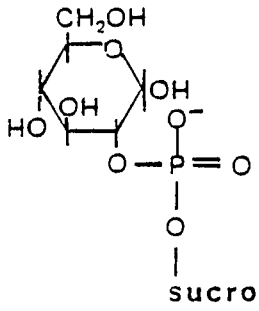
Figure 4.3 shows that this data can be explained if the phosphate in Agrocinopine C is linked to the hydroxyl group on carbon atom 2 of D-glucose. Under alkaline conditions the sucrose molecule is readily lost from Agrocinopine C and the phosphate cyclises to form a mixture of the cyclic 1, 2 and 2, 3 phosphates. The cyclic phosphates are not resolved on H.V.P.E., and thus only one spot is seen. Further ammonolysis of Agrocinopine C cleaves the cyclic phosphates yielding a mixture of G-1-P, G-2-P and G-3-P.

4.6.3 Acid hydrolysis time course.

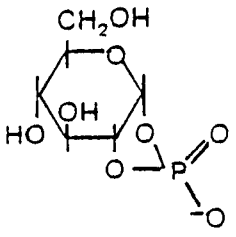
Further information on the location of the phosphate linkages was obtained from the acid hydrolysis time course of Agrocinopine C (Figure 3.4). Acid hydrolysis of Agrocinopine C proceeds in two steps, the first stage is virtually complete within 2 hours, followed by a much slower cleavage which does not reach completion until 6 hours. From the work done in the synthesis of the glucose phosphates, it had become apparent that G-1-P, G-2-P and G-3-P were easily cleaved under acid conditions while G-6-P and G-4-P were much more difficult to hydrolyse.

FIGURE 4.3 CYCLISATION OF AGROCINOPINE C UPON AMMONOLYSIS.

Ammonolysis of Agrocinopine C leads first to the loss of the sucrose molecule. Under alkaline conditions, the phosphate group then cyclises to form the cyclic glucose 1 2 phosphate and the cyclic glucose 2 3 phosphate. Further ammonolysis cleaves the cyclic phosphates to yield glucose-1-phosphate, glucose-2-phosphate and glucose-3-phosphate.



AGROCINOPINE C



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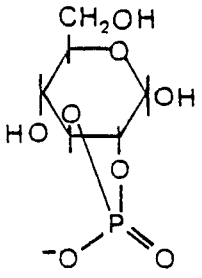
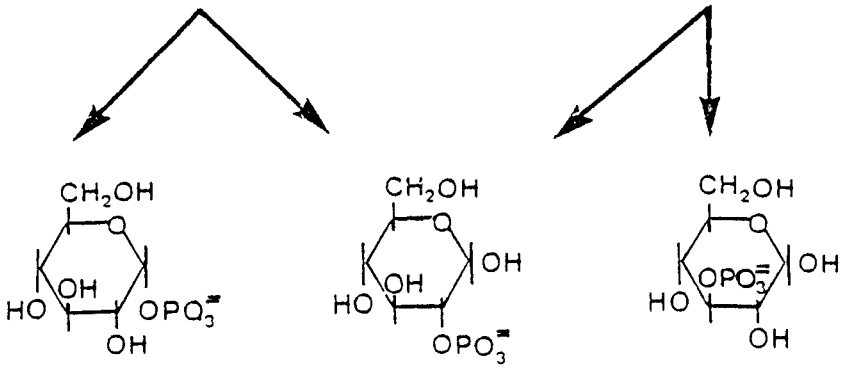


cyclic glucose 1,2 phosphate cyclic glucose 2,3 phosphate



glucose-1-PO_4 glucose-2-PO_4 glucose-3-PO_4

Comparison of the Agrocinopine C hydrolysis rate (Figure 3.4), with that of G-2-P and G-6-P revealed that G-2-P hydrolysed under acid conditions in a manner which was similar to the first step in the Agrocinopine C hydrolysis, while G-6-P underwent a much slower cleavage similar to the second step in the acid hydrolysis of Agrocinopine C.

4.7 ^{13}C-NMR

^{13}C-NMR spectroscopy is dependent on the fact that the 13C nucleus behaves as a bar magnet and when subjected to an external magnetic field the nucleus is induced to spin and will resonate with a particular frequency of an applied radio-frequency signal. This frequency is characteristic of a particular element in a given magnetic field. The chemical environment in which the nucleus resides will alter its resonance energy, thus leading to a unique "chemical shift" for most nuclei, and, providing a sufficiently powerful magnetic field is present, each carbon atom will be resolved in the spectrum.

3 (Trimethyl silyl) propionic acid d4 (T.P.S.) has been used as an internal reference standard and the chemical shift of its methyl groups is arbitarily designated zero. The chemical shift of the sample of ^{13}C is then defined in terms of the dimensionless δ value where

$$\delta = \frac{V_S - V_{TPS}}{\text{operating freq.}} \times 10^6 \text{ ppm.}$$

In general, highly oxygenated carbons have a large chemical shift and their resonance peaks will be seen at the left hand end of the spectrum, furthest from the TSP methyls, while the most hydrogenated carbons will have a low chemical shift, appearing at the right hand end of the spectrum and nearest the TSP methyls. Thus in sugar molecules the anomeric carbon (C1) being the most highly oxygenated carbon appears at the left of the spectrum while primary carbons (C_6) appear to the right of the spectrum. In carbohydrates the majority of the carbons are secondary hydroxyls and therefore appear between these two extremes.

Figure 3.7 and Table 3.8 show the ^{13}C-NMR spectra of the standard compounds, glucose-6-phospate, D-glucose and glucose-1-phosphate. The carbon assignments for D-glucose were taken from published data (Gorin and Mazurek 1975). Those of glucose-6-phosphate and glucose-1-phosphate were made by comparison with D-glucose. In both the D-glucose spectrum and the glucose-6-phosphate spectrum, each carbon gives rise to two signals.

These pairs of signals correspond to the two possible orientations of the pyranose anomeric carbon, α and B. Glucose-1-phosphate gives rise to only the αform since the anomeric carbon is phosphorylated and only one isomer is present.

In glucose-6-phosphate the 6th carbon has undergone a shift to left of the spectrum and appears as a

doublet peak. This shift to the left and splitting of the resonance signal is quite characteristic of phosphorylated carbons. The carbon atom immediately adjacent to the phosphorylated carbon (C5) has undergone an up-field shift, and this too is characteristic of carbon atoms adjacent to phosphorylated carbons. The splitting of the carbon signal is measured by the "coupling constant" (J). In this case $^{2}J_{poc}$ = 3.4 Hz.

The G-1-P spectrum shows a similar down-field shift and a doublet peak in the anomeric carbon signal. The coupling constant $^{2}J_{poc}$ = 6.11 Hz. The 2nd carbon nucleus shows a shift upfield to the right and becomes resolvable from the carbon 5 peak.

Figure 3.8 and Table 3.9 show the ^{13}C-NMR spectrum obtained for Agrocinopine C. Also shown are the spectra for D-glucose and sucrose. Again the carbon assignments for D-glucose were taken from published literature (Gorin & Mazurek 1975). Carbon assignments for Agrocinopine C have been made by comparison with the spectrum for D-glucose and sucrose and also by comparison with the spectrum of Agrocinopine A (Figure 3.9).

It can be seen that the anomeric carbons (F2s, G1g and G1s) appear at the left of the spectrum and the primary carbons (F6s, F1s, G6s and G6g) appear at the right end of the spectrum. In general the carbon signals in Agrocinopine C "line up" well with

their counterparts in the glucose or sucrose spectrum.

There are however some major changes which are indicated in the figure with dotted lines. The carbon-2 corresponding to the second carbon in the free glucose molecule in Agrocinopine C has undergone a large shift down-field and appears as a doublet signal (^{2}J poc = 1.88), while carbon 1 and carbon 3 (again from the free glucose molecule in Agrocinopine C) have shifted to the right, and also appear as doublet signals (^{3}J pocc = 0.87 and 6.1) respectively. This pattern is characteristic of phosphorylation of the 2nd carbon in the free glucose molecule of Agrocinopine C.

Carbon 6 from the glucose moiety of the sucrose portion of Agrocinopine C (G6s) has also shifted down-field and appears as a doublet signal (J poc = 3.6). Thus the carbon 6 in the glucose moiety of the sucrose molecule in Agrocinopine C appears to be phosphorylated.

Comparison of the ^{13}C-NMR spectrum of Agrocinopine C and Agrocinopine A are shown in Figure 3.9 and Table 3.10. Several major differences in these spectra illustrate the chemical differences between the two molecules. Agrocinopine A exhibits resonance and peaks corresponding to both the α and β forms of the arabinose portion of the molecule. These peaks are not seen in Agrocinopine C, but instead, peaks corresponding to the free glucose molecule are shown.

The other major difference between the spectra is in the position of the carbon atoms 3, 4 and 5 of the fructose molecule. In Agrocinopine A, carbon 4 from the fructose (F4s) has a large shift to the left and is present as a doublet peak (^{2}J poc = 4.9), while the adjacent carbons F5s and F3s have shifted upfield and also appear as doublet peaks (^{3}J pocc = 4.9and 4.9 respectively). These differences reflect the fact that in Agrocinopine A the phosphodiester linkage is through the 4th carbon in the fructose molecule, while in Agrocinopine C this linkage occurs through the sixth carbon of the glucose moiety. As would be expected, Agrocinopine A does not show the shift or splitting of the 6th carbon in the glucose portion of the sucrose molecule (G6s) which appears in the Agrocinopine C spectrum.

Thus the ^{13}C-NMR data supports the findings of the degradative studies, and together this experimental evidence has led to the proposed structures of Agrocinopine C and D shown in Figure 4.1.

4.8 COMPARISON OF CHEMICAL STRUCTURES OF THE AGROCINOPINES A, B, C AND D.

A comparison of the chemical structures of Agrocinopines A, B, C and D are shown in Figure 4.4. As can be seen, the Agrocinopines are closely related, showing only 2 major differences:

(1) The free sugar in Agrocinopine A and B consists of an arabinose molecule, while Agrocinopines C and D contain a free D-glucose molecule.

(2) The second major difference is that Agrocinopine A is linked through the fructose moiety of the sucrose portion and consequently Agrocinopine B has lost a glucose molecule while Agrocinopine C is linked through the glucose portion of the sucrose molecule and thus Agrocinopine D has lost a fructose molecule. Thus in the case of sensitive strains of Agrobacterium such as K476 the nature of the sucrose phosphodiester is apparently not a feature of importance in the biological activity of these compounds.

The overlay on Figure 4.3 shows that three carbons (C1, C2 and C3 of the free sugar) and the phosphate grouping are identical in all of the Agrocinopines. This is particularly interesting in view of the fact that arabinose-2-phosphate is biologically active (M. Ryder unpublished data) in the bioassay system used to detect Agrocinopine A.

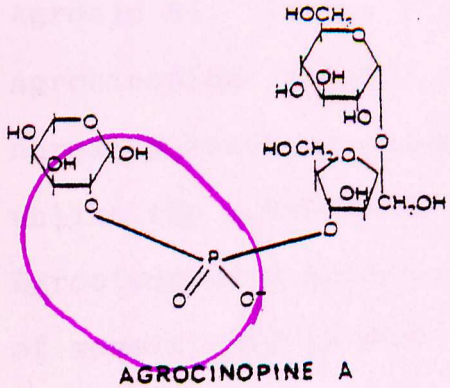
Data shown here (Figure 3.14) shows that glucose-2-phosphate is also biologically active, counteracting the toxic effect of Agrocin 84 on the normally sensitive strain K476.

This would suggest that it is the phosphorylated free sugar portion of the Agrocinopines, in particular the three carbons marked on Figure 4.3 which are

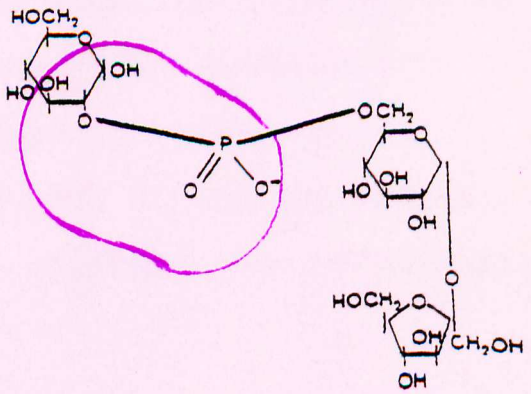
FIGURE 4.4 COMPARISON OF THE CHEMICAL STRUCTURES OF THE AGROCINOPINES.

The overlay illustrates the three carbons, and the phosphate groupings which are identical in all four molecules.

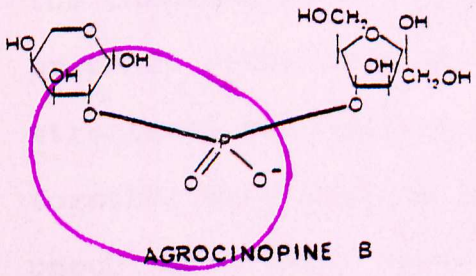
AGROCINOPINE C



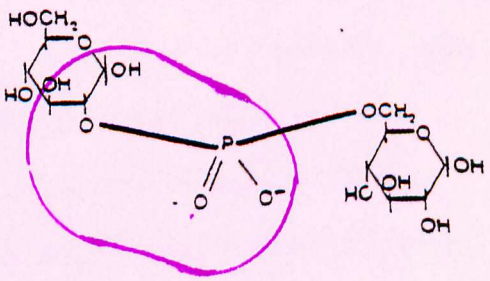
AGROCINOPINE A



AGROCINOPINE D



AGROCINOPINE B



recognised by the permease allowing their entry into the pathogenic Agrobacterium Ti host cell.

The biological activity of the phosphorylated sugars (arabinose-2-phosphate and glucose-2-phosphate) can be explained in terms of competition with Agrocin 84 for this permease (Ellis & Murphy 1981).

Glucose-2-phosphate did not induce the normally insensitive strain K478 to become sensitive to Agrocin 84. If the induced sensitivity conferred by Agrocinopine C and D is due to derepression of normally inactive genes, then it would seem that unlike the constituent permease of K476 the entire Agrocinopine D molecule is required for the induction of sensitivity in K478.

In conclusion, the work presented here establishes the chemical nature of Agrocinopine C and D and their proposed structures can be seen in Figure 4.3. The structural elucidation of Agrocinopine C and D, together with that of Agrocinopine A and B (Ryder unpublished data) opens the way for further studies on the bio-synthetic pathways for the Agrocinopines and the molecular basis of their interactions with Agrocin 84.

APPENDIX 1

Composition of Media

Stonier's Medium (Stonier, 1960b)

$CaSO_4$	0.1 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.2 g
NH_4NO_3	2.7 g
$FeNO_3$	5.0 mg
$MnCl_2$	0.1 mg
$ZnCl_2$	0.1 mg
Potassium Citrate	10.0 g
Sodium Glutamate	2.0 g
NaH_2PO_4	0.3 g
K_2HPO_4	0.88 g
Biotin	2.0 ug
Thiamine	4.0 mg) Add filter steri-
	) lized after
Calcium Pantothenate	4.0 mg) autoclaving
Distilled H_2O	to 1 litre
Agar	1.5 g (for solid medium)

Yeast Mannitol Agar

K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.2 g
$CaCl_2$	0.2 g
$FeCl_3$	0.01 g
Yeast Extract	1.0 g
Mannitol	10.0 g
Agar	15.0 g
Distilled H_2O	to 1 litre

Buffered Agar

Na_2HPO_4	17.3 g
NaH_2PO_4	12.1 g
Agar	8.0 g
Distilled H_2O	to 1 litre

REFERENCES

Avela, E., Aspeluno, S., Holmbom, B., Melander, B., Jalonen, H., and Peltonen, C. Selective Substitution of Sucrose Hydroxyl Groups via Chelates. A.C.S. Symposium Series 41 (1977).

Bartlett, G.R. Phosphorous Assay in Column Chromatography. J.Biol.Chem. 234, 466-468 (1959).

Bergmeyer, H.U., Gawehn, K., Grassl, M. "Enzymes as Biochemical reagents" in Methods of Enzymatic Analysis Vol. 1. Ed. H.U. Bergmeyer, pp.457-458 Verlag Chemie Weinheim Academic Press Inc. N.Y. and London, 1977.

Braun, A.C. (1947). Thermal studies on the factors responsible for Tumour Initiation in Crown Gall. Amer.J.Bot. 34, 234-240.

Braun, A.C. and White, A.C. A Cancerous Neoplasm of Plants. Autonomous bacteria-free Crown Gall Tissue. Cancer Res. 2, 597-617 (1942).

Brown, D., Hayes, M. and Todd, M. Der Abbau Von Glucose -3-Phosphat Durch Alkali. Chemische Berichte Janrg. 90, 936-941 (1957).

Chilton, M.D., Drummond, M.H., Merlo, D.J., Sciaky, D., Montoya, A.L., Gordon, M.P., and Nester, E.W. Stable Incorporation of Plasmid DNA into Plant Cells: The Molecular basis of Crown Gall Tumorigenisis. Cell 11, 263-271 (1977).

Chilton, M.D., Montoya, A.L., Merlo, D.J., Drummond, M.H., Nutter, R., Gordon, M.P. and Nester E.W. Restriction Endonuclease Mapping of a Plasmid that confers Oncogenicity upon Agrobacterium tumefaciens strain B6-806. Plasmid 1, 254-269. (1978).

Chilton, M.D., Saiki, R.K., Yadau, N., Gordon, M.P. and Quetier, F. T-DNA of Agrobacterium Ti plasmid is in the Nuclear DNA Fraction of Crown Gall Tumour Cells. Proc. Natl.Acad.Sci. U.S.A. 77, 4060-4064 (1980).

Chilton, M.D. A vector for introducing new genes into plants. Scientific American 248, 36-46 (1983).

Depicker, A., Van Montagu, M., and Schell, J. Homologous DNA Sequences in different Ti Plasmids are essential for Oncogenicity. Nature (London) 275, 150-153 (1978).

Depicker, A., De Wilde, M., De Vos, G., De Vos, R., Van Montagu, M. and Schell, J. Molecular Cloning of Overlapping Segments of the Nopaline Ti-plasmid pTi (58) as a means to restriction Endonuclease Mapping. Plasmid 3, 193-211 (1980).

Drummond, M.H., Gordon, M.P., Nester, E.W. and Chilton, M.D. Foreign DNA of Bacterial Plasmid Origin is transcribed in Crown Gall Tumours. Nature (London) 269, 535-536 (1977).

Dubois, M., Gilles, K., Hamilton, J.K., Rebers, P.A., and Smith, F. A colorimetric Method for the determination of sugars. Nature 168, 167 (1951)

Ellis, J.G. Genetic Aspects of the Induction and Biological Control of Crown Gall. PhD Thesis, Waite Agricultural Research Institute, University of Adelaide. Nov. 1980.

Ellis, J.G. and Murphy, P.J. Four New Opines from Crown Gall Tumours - Their Detection and Properties. Molec. Gen. Genet. 181, 36-43 (1981).

Firmin, J.L. and Fenwick, G.R. Agropine - A major new Plasmid - Determined Metabolite in Crown Gall Tumours. Nature (London) 276, 842-844. (1978).

Garfinkel, D.J. Genetic Analysis of Crown Gall : A fine Structure Map of the T-DNA by Site Directed Mutacens. Cell, 27. 143-153 (1981).

Goldman, A., Tempe, J. and Morel, G. Quelques particuliarites De Diverses Souches d'Agrobacterium tumefaciens. C.R.Soc. Biol. 162, 630-631 (1968).

Goldman, A., Thomas, A.W., and Morel, G. Sur la Structure de le Nopoline Metabolite Anormale de Certaines Tumeurs de Crown Gall. C.R.Acad.Sc.Paris 268D, 852-854 (1969).

Gorin, P.A.J. and Mazurek, M. Further studies on the Assignment of Signals in ^{13}C Magnetic Resonance Spectra of Aldoses and Derived Methyl Glycosides Can.J.Chem. 53, 1212-1222 (1975).

Guyon, P., Chilton, M.D., Petit, A. and Tempe, J. Agropine in 'Null Type' Crown Gall Tumours: Evidence for generality of the Opine Concept. Proc.Nat. Acad.Sci. U.S.A. 77, 2693-2697 (1980).

Hamilton, R.H. and Fall, M.Z. The loss of tumour-initiating ability in Agrobacterium tumefaciens by incubation at high temperatures. Experientia 27, 229-230 (1971).

Hernalsteens, J.P. Identification of Ti-plasmid functions by Transposon Mutagenisis. Mapping of some functions of an Octopine Ti plasmid. Proc. 4th Int. Conf. Plant Path. Bacteria P.260 Angers (1978).

Hernalsteens, J.P., Van Vliet, F., DeBeuckeleer, M., Depicker, A., Engler, G., Lemmers, M., Holsters, M., Van Montagu, M. and Schell, J.
The Agrobacterium tumefaciens Ti plasmid as a host vector system for introducing foreign DNA in plant cells. Nature (London) 287, 654-656 (1980).

Holsters, M., Silva, B., Van Vliet, F., Genetello, C., De Block, M., Dhaese, P., DePicker, A., Inze, D., Engler, G., Villarroel, R., Van Montagu, M., and Shchell, J. The functional organization of the Nopaline Agrobacterium tumefaciens Plasmid pTi C58. Plasmid 3, 212-230 (1980).

Hough, L., Jones, J.K.N. and Wadman, W.H.
Quantitative Analysis of mixtures of sugars by the method of partition chromatrography;Part V Improved methods for the separation and detection of the sugars and their methylated Derivatives on paper chromatogram. J.Chem.Soc. 1702-1703 (1950).

Kerr, A. Transfer of Virulence between Isolates of Agrobacterium. Nature (London) 223, 1175-1176 (1969).

Kerr, A. Acquisition of Virulence by Non-Pathogenic Isolates of Agrobacterium Radiobacter. Physiol. Pl. Pathol. I, 241-246 (1971).

Kerr, A. and Htay, K. Biological Control of Crown Gall through bacteriocin production. Physiol.Pl.Pathol. 4, 37-44 (1974).

Kerr, A. and Roberts, W.P. Agrobacterium : Correlations between and transfer of Pathogenecity, Octopine and Nopaline Metabolism and bacteriocin 84 sensitivity. Physiol.Pl.Pathol. 9, 205-211 (1976).

Kerr, A. The Ti plasmid of Agrobacterium. Proc. 4th Int. Conf. Plant Path. Bact. Angers (1978).

Klapwijk, P.M., Scheulderman, T. and Schilperoort, R.A. Co-ordinated Regulation of Octopine Degradation and Conjugative Transfer of Ti plasmids in Agrobacterium tumefaciens. Evidence for a common regulatory gene and separate operons. J.Bacteriol. 136, 775-785 (1978).

Koekman, B., Ooms, G., Klapwijk, P. and Schilperoort, R. Genetic Map of an Octopine Ti plasmid. Plasmid 2, 347-357 (1979).

Leemans, J., Shaw, C., Deblaere, R., DeGreve, H., Hernalsteens, J.P., Maes, M., Van Montagu, M. and Schell, J. Site-specific mutagenis of Agrobacterium Ti plasmids and transfer of genes to plant cells. J.Mol.Appl.Genet. 1, 149-164 (1981).

Menage, A. and Morel, G. Sur la Presence D'Octopine Dans les tissus de Crown Gall Cultives. C.R.Acad.Sci. Paris 259, 4795-4796 (1964).

Montoya, A.L., Chilton, M.D., Gordon, M.P., Sciary, D. and Nester, E.W. Octopine and Nopaline Metabolism in Crown Gall Tumour : Role of Plasmid Genes. J.Bacteriol. 129, 101-107 (1977).

Murphy, J. and Riley, P. A modified single solution method for the determination of phosphate in natural waters. Anal.chim Acta 27, 31-36 (1962).

Murphy, P.J. and Roberts, W.P. A basis for Agrocin 84 Sensitivity in Agrobacterium Radiobacter. J.Gen. Microbiol. 114, 207-213 (1979).

Ooms, G., Klapwijk, P.M., Poulos, J.A. and Schilperoort, R.A. Characterization of Tn 904 Insertion in Octopine Ti plasmid mutants of Agrobacterium tumefaciens. J.Bacteriol. 144 : 82-91 (1980).

Petit, A., Delhaye, S., Tempe, J. and Morel, G. Recherches sur les Guanidines Des Tissus De Crown Gall. Mise en Evidence D'une Relation biochimique specifique entre les souches d'Agrobacterium tumefaciens et les tumeurs Qu'elles indusent. Physiol. Veg. 8, 205-213 (1970).

Petit, A., Dessaux, Y. and Tempe, J. The biological significance of Opines : I. A study of Opine Catabolism by Agrobacterium tumefaciens. Proc. 4th Int. Conf. Plant Path. Bacteria pp.143-152. Angers (1978).

Schell, J., Van Montagu, M., DeBeuckeleer, M., DeBlock, M., DePilker, A., DeWilde, M., Engler, G., Genetello, C., Hernalsteens, J.P., Holsters, M., Seuринck, J., Silva, A., Van Vliet, F. and Villarroel, R. Interactions and DNA transfer between Agrobacterium tumefaciens, the Ti plasmid and the Plant Host. Proc.R.Soc. London B204, 251-266 (1979).

Sciaky, D., Montoya, A.L. and Chilton, M.D. Fingerprints of Agrobacterium Ti plasmids. Plasmid I. 238-253 (1978).

Smith, E.F. and Townsend, C.O. A plant tumor of bacterial origin. Science, N.Y. 25, 671-673. (1907).

Tate, M.E. Separation of Myoinositol Penta-phosphates by moving paper electrophoresis. Anal. Biochem. 23, 141-149 (1968).

Tate, M.E., Murphy, P.J., Roberts, W.P. and Kerr, A. Adenine N^6-substituent of Agrocin 84 determines its bacteriocin-like specificity. Nature (London) 280, 697-699 (1979).

Trevelyan, W.E. Procter, D.P. and Harrison, J.S. Detection of sugars on Paper chromatograms. Nature 166, 444-445 (1950).

Van Larebeke, N., Engler, G., Holsters, M., Van Den Elsacker, S., Zaenen, I., Schilperoort, R.A. and Schell, J. Large plasmid in Agrobacterium tumefaciens essential for Crown Gall-inducing ability. Nature (London) 252, 169-170 (1974).

Watson, B., Currier, T.C. Gordon, M.P., Chilton, M.D. and Nester, E.W. Plasmid required for virulence of Agrobacterium faciens. J. Bacteriol. 123, 255-264 (1975).

Zambryski, P., Holsters, M., Kruger, K., DePicker, A., Schell, J. and Van Montagu, M. Tumor DNA structure in Plant Cells transformed by Agrobacterium tumefaciens. Science 209, 1385-1391 (1980).