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The ability of N,N'-diphenylurea (DPU) to substitute for cytokinin-active adenine derivatives in promoting callus growth of Phaseolus lunatus and Nicotiana tabacum has been examined. In general, DPU stimulated callus growth of P. lunatus at high concentrations and the growth of most callus tissues was irregular, while the response of tobacco callus tissue to DPU was more uniform. Genotypic variations in sensitivity and uniformity of callus growth in response to DPU were found among different genotypes of P. lunatus.

Tissues cultured on DPU-containing medium for one passage acquired the ability to proliferate in subsequent passages in the absence of either DPU or cytokinin-active adenine derivatives. Corresponding P. lunatus callus tissues maintained on optimal concentration of kinetin and tobacco callus tissue grown on DPU-containing medium remained cytokinin-dependent. However, P. lunatus callus tissues grown on media containing suboptimal or supraoptimal concentrations of kinetin also exhibited cytokinin-autonomous growth when transferred to cytokinin-

free media.

In studies of the interaction of kinetin and DPU in promoting the growth of P. lunatus callus tissues, it was observed that the effects of kinetin and DPU in promoting callus growth were not additive and that the effect of increasing kinetin concentrations in suppressing the development of the cytokinin-autonomous growth habit was overcome to some extent by the presence of DPU in the culture medium.

N^6-ureidopurines also stimulated vigorous and uniform callus growth of P. lunatus genotypes and the behavior of these callus tissues when transferred to cytokinin-free media was similar to that of callus tissues grown on either benzylaminopurine or kinetin. Optimal concentrations of these compounds suppressed the development of cytokinin autonomy. The use of stronger auxin than indole-3-acetic acid in the tobacco tissue culture media did not significantly alter the response of tobacco callus tissue to DPU.

Methodology for the detection of cytokinin biosynthesis (from adenine-8-^{14}C) in callus tissue has also been examined. Model experiments using Amberlite XAD-2 columns demonstrated the effectiveness and simplicity of the use of this material in preliminary purifications of naturally occurring cytokinins and in the reduction of background label in experiments to detect cytokinin biosynthesis from adenine-8-^{14}C. The recovery of cytokinin activity from tRNA hydrolysates and from standard solutions applied to Amberlite XAD-2 column has been tested. In addition, the effectiveness of this material in the initial purification of cytokinins from Agrobacterium tumefaciens culture filtrate and from callus tissue of P. lunatus has been examined.

CONTROL OF CYTOKININ AUTONOMY IN PHASEOLUS LUNATUS CALLUS CULTURES

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my wife and two daughters

Hye-Yeon, Hye-Jeen

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ABBREVIATIONS AND DEFINITIONS

A_{260} unit : that amount of material which has an absorbance of 1 when dissolved in 1 ml solvent and measured at 260 nm with a 1-cm light path

CMP : N^6-cyclohexylmethylaminopurine

CPM : Counts per minute

CUP : N^6-cyclohexylureidopurine

cv : cultivar

Cytokinins :

bzl^6Ade or BAP : N^6-benzyladenine

fr^6Ade : N^6-furfuryladenine, kinetin

i^6Ade : 6-(3-methyl-2-butenylamino)purine or N^6-(Δ^2-isopentenyl)-adenine

ms^2i^6Ade : 6-(3-methyl-2-butenylamino)-2-methylthiopurine

io^6Ade : 6-(4-hydroxy-3-methyl-2-butenylaminno)purine, zeatin

ms^2io^6Ade : 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-purine, methylthiozeatin

The 9-ß-D-ribosides of the above cytokinins are indicated by the substitution of A for Ade.

2,4-D : 2,4-dichlorophenoxyacetic acid

DPU : N,N'-diphenylurea

IAA : indole-3-acetic acid

KE : kinetin equivalents-the µg of kinetin required to give the same activity as the test sample under the specified conditions

P.I. line : plant introduction line

picloram : 4-amino-3,5,6-trichloropicolinic acid

PUP : N^6-phenylureidopurine

tRNA : transfer ribonucleic acid

TUP : N^6-p-tolylureidopurine

CONTROL OF CYTOKININ AUTONOMY IN *PHASEOLUS LUNATUS* CALLUS CULTURES

I. INTRODUCTION

A factor promoting cell division in tobacco pith tissue was isolated from an autoclaved sample of herring sperm DNA and characterized as 6-furfurylaminopurine (kinetin) by Miller *et al.* in 1955. Subsequently, several synthetic and naturally occurring N^6-substituted adenine derivatives were shown to exhibit biological activity similar to that of kinetin. Skoog *et al.* (1965) proposed the generic name "cytokinin" for all compounds that induce cell division and exert certain other growth regulatory functions similar to that of kinetin. At the present time, the number of known synthetic cytokinins has increased to several hundred (Kende, 1971), and more than twenty naturally occurring cytokinins have been identified (Skoog and Armstrong, 1970; Kende, 1971; Hall, 1973).

The most active cytokinins that have been synthesized or identified as naturally occurring compounds are N^6-substituted adenine derivatives. In the tobacco callus bioassay, compounds with an intact adenine nucleus and an N^6-isoprenoid side chain possess the highest cytokinin activity (Skoog and Armstrong, 1970). However, it has been known for some time that certain compounds that are not N^6-substituted adenine derivatives, urea and thiourea derivatives (*e.g.* N,N'-diphenylurea), also exhibit weak cytokinin-like activity in various bioassay systems (Shantz and Steward, 1955; Bruce, *et al.*, 1965; Bruce and Zwar, 1966; Karanov *et al.*,

1968). More recently, the synthesis of substituted urea derivatives with cytokinin activity approaching or exceeding that of the most active adenine derivatives in promoting the growth of tobacco tissue cultures (Okamoto, et al., 1974; Takahashi et al., 1978), and in stimulating shoot formation in the callus cultures (Isogai et al., 1976; Okamoto et al., 1978) has been reported. The similarity in the biological activity of two such structurally unrelated classes of compounds as N^6-substituted adenine derivatives and the cytokinin-active urea derivatives poses one of the more interesting problems in cytokinin structure-activity relationships.

The effects of N,N'-diphenylurea on the growth of cytokinin-dependent tissue cultures of Phaseolus lunatus have been examined here and compared with the effects of this compound on the growth of tobacco callus tissue (Nicotiana tabacum cv. Wisconsin 38). This study was undertaken as part of a program to detect intrinsic genetic variation in cytokinin metabolism in Phaseolus. The effects of N,N'-diphenylurea on tissue cultures derived from a number of P. lunatus genotypes have been tested in the present study, and the biological response to this compound has been examined in detail in selected genotypes.

Preliminary studies concerning the effect of N,N'-diphenylurea on cytokinin metabolism in tobacco callus tissue have also been undertaken.

II. LITERATURE REVIEW

A. The cytokinin activity of urea derivatives.

Shantz and Steward (1952) isolated from coconut milk three factors which stimulated cell division in carrot tissue cultures. One of these compounds was subsequently identified as N,N'-diphenylurea (Shantz and Steward, 1955). The effect of diphenylurea in promoting callus growth was variable, apparently depending on the carrot from which their assay explants were taken. Subsequently, DPU and related synthetic compounds were shown to exhibit biological activity similar to that of kinetin in various cytokinin bioassay systems (Strong, 1958; Miller, 1960; Bruce et al., 1965; Kefford et al., 1965, 1966; Bruce and Zwar, 1966).

In the tobacco callus bioassay for cytokinins, DPU is active only at concentrations much higher than that of the more active N^6-substituted adenine derivatives (Strong, 1958; Miller, 1960; Buttomley et al., 1963). In addition to promoting cell division, certain urea derivatives are also active in promoting the development of pea lateral buds (Bruce et al., 1965; Kefford et al., 1966), in the retardation of senescence in radish leaf discs (Bruce et al., 1965; Kefford et al., 1973), in the promotion of lettuce seed germination (Bruce et al., 1965; Kefford et al., 1965), and in the alteration of sex expression in Luffa acutangula (Bose and Nitsch, 1970).

The relationship between cytokinin activity and the chemical structure of substituted urea derivatives was investigated by Bruce

and Zwar (1966). These investigators synthesized and tested the cytokinin activity of approximately 500 derivatives of urea and thiourea, mainly of the N-monosubstituted and N,N'-disubstituted types, in the tobacco callus bioassay. The simplest active compound of those tested was N-phenylurea. The introduction of substituents into the benzene ring of N-phenylurea increased the activity of the compound. Compounds substituted in the meta-position were the most active, while those substituted in the ortho-position possessed lowest activity. Substances with electron-accepting substituents were more active than those with electron donating substituents. In the N,N'-disubstituted urea series, those compounds that possessed one unsubstituted benzene ring had the highest activity. Compounds with two substituted rings always had low activity. Compounds in which both amino hydrogen atoms on one or both sides of the bridge were replaced by other groups were inactive or exhibited reduced activity. However, all of the urea derivatives tested were less active than kinetin. Kinetin exhibits detectable activity in this bioassay at a concentration of 0.01 μM, while the most active urea derivatives tested exhibited weak and variable activity at 0.4 μM. Similar structure-activity relationships were observed in the chlorophyll retention test with radish leaf discs, except for the case of N-4-pyridylurea, which was active in chlorophyll retention and inactive in promoting cell division in the tobacco bioassay (Kefford et al., 1973). Some urea derivatives (including DPU) were highly active in the chlorophyll retention test and exhibited activity comparable to that of kinetin. A -NH-CO-NH- bridge and a

planar aromatic ring appeared to be required for the activity of urea derivatives in both the radish leaf disc and tobacco callus bioassays. On the basis of these results, it was suggested that the receptor site for cytokinin activity was the same in both senescence retardation and in the promotion of cell division.

Kefford, Zwar and Bruce (1965) investigated the effect of a number of urea derivatives, on the germination of lettuce seeds. They found considerable differences between the structural requirements for activity in this test and in the tobacco callus bioassay. For example, 2,4- and 2,6-dimethylphenylurea were active in promoting lettuce seed germination, but inactive in promoting cell division in tobacco callus tissue. Thiourea was inactive in the tobacco callus bioassay but active to some extent (at concentrations of about 10^{-4} to 10^{-2} M) in promoting germination. Thus, the activity of urea derivatives in promoting seed germination was not necessarily correlated with activity in promoting cell division. It is possible that the promotion of lettuce seed germination by urea derivatives and thiourea is not directly related to cytokinin activity in all cases. Karanov et al. (1968) extensively investigated the activity of thiourea derivatives in retarding chlorophyll loss and in promoting the growth of radish leaf discs. High cytokinin activity in the chlorophyll retention test was possessed by N-allyl-N'-hydroxy-4-carboxyphenylthiourea. This compound was 80% as effective as kinetin when the two compounds were compared at a concentration of 10^{-5} M, but at higher concentrations, kinetin was more effective. N-phenyl-N'-p-carboxyphenylthio-

urea promoted the enlargement of radish leaf discs at a concentration of 5 x 10^{-6} M, which was five times lower than the kinetin concentration required to give detectable activity. The response of the discs to this thiourea derivative was only 10% less than that obtained with kinetin. In contrast to the results obtained by Kefford et al. (1965) in which thiourea was inactive in promoting cell division of tobacco tissue culture, Erez (1977) reported that thiourea promoted callus growth in soybean, tobacco, and apple tissue cultures at concentrations of 10^{-4} to 10^{-3} M. The effect of thiourea on soybean callus growth was examined in the presence of various concentrations of cytokinins, and a pronounced synergistic effect was observed at all cytokinin levels tested. The synergism was particularly marked when zeatin, rather than kinetin and N^6-benzyladenine, was used as the cytokinin. The calculated cell number per callus as well as the fresh weight of tissue grown on thiourea-containing medium was only one-third of that of tissue grown on zeatin-containing medium, but callus tissues grown on thiourea and zeatin combinations exhibited a two-fold increase in fresh weight and in cell number per callus compared with callus tissue grown on zeatin-containing medium. On the basis of these results, it was suggested that mode of action of thiourea in promoting cell division is different from that of the purine cytokinins and also different from that of other urea and thiourea derivatives. The sulfhydryl group of thiourea appears important to its activity.

Recently, it has been shown that N-(4-pyridyl)-N'-phenylurea, and its derivatives, have high cytokinin activity in promoting tobacco

callus growth (Okamoto et al., 1974; Takahashi et al., 1978). Among these compounds, N-phenyl-N'-(2-chloro-4-pyridyl)urea was the most active, giving detectable activity at concentrations as low as 4×10^{-9} M. Generally, every substitution on the phenyl ring of the parent molecule, N-(4-pyridyl)-N'-phenylurea, decreased biological activity and gave products with activities in the order: meta > ortho > para. In addition, these investigators (Isogai et al., 1976; Okamoto et al., 1978) reported that pyridylphenylurea and its derivatives enhanced organ formation in tobacco callus tissue.

Certain N-benzylurea derivatives, which are inactive or weakly active in chlorophyll retention and in other bioassays for cytokinin activity, have been reported to antagonize the effects of both cytokinin-active urea derivatives and N^6-substituted adenine derivatives in retarding the senescence of radish leaf discs (Kefford et al., 1968). The presence of N-benzyl-N'-3,4-dichlorophenylurea at 5×10^{-6} M inhibited the action of both kinetin and N-3,4-dichlorophenylurea in retarding the senescence of radish leaf discs. Similar effects were observed using N-benzyl-N'-phenylurea, N-benzyl-N'-4-bromophenylurea, and N-benzyl-N'-4-fluorophenylurea at a concentration of 2.5×10^{-5} M. On the basis of these results, it was suggested that purine cytokinins and cytokinin-active urea derivatives act at the same site within the cell. The phenyl ring of N-benzyl-N'-phenylurea may be the part of the molecule that binds to the hormonal receptor site, because N-benzylurea itself is not an antagonist.

Miller (1961) suggested that DPU might serve as a precursor of

a kinetin-like compound, possibly phenylaminopurine or phenylureidopurine. Several ureidopurines have been synthesized and found to possess cytokinin activity in the tobacco callus bioassay (McDonald et al., 1971) and in promoting the growth of soybean tissue culture (Dyson et al., 1972; Hong et al., 1973). The compound N-[9-(ß-D-ribofuranosyl-9H)-purin-6-ylcarbamoyl]threonine (a ureidopurine) does occur as a constituent of certain tRNA species responding to codons with the initial letter A (Chheda et al., 1969; Schweizer et al., 1969; Yamada et al., 1969) and in culture filtrates from Rhizopogon roseolus (Laloue and Hall, 1973), but no evidence has been obtained to date for the conversion of DPU to a purine derivative. Furthermore, the structure-activity relationships exhibited by ureidopurines (McDonald et al., 1971) are not similar to those observed for urea derivatives. The substituted 6-phenylureidopurines exhibited decreasing activity in the order o-tolyl > m-chlorophenyl > p-tolyl side chain. This contrasts with the order: m-chlorophenyl > p-tolyl > o-tolyl reported by Bruce and Zwar (1965) in tests of N-phenylurea derivatives in the tobacco callus bioassay.

Dyson et al. (1972) have suggested that the ability of DPU to promote cell division in tobacco callus tissue may be related to the ability of their tissue to metabolize DPU. They observed three unidentified radioactive metabolites in an ethanol extract from tobacco callus tissue supplied with ^{14}C-labelled DPU. Soybean callus tissue did not respond to DPU and only the parent molecule (radiolabelled DPU) was recovered in an ethanol extract from soybean callus tissue

supplied with DPU.

Burrows and Leworthy (1976) isolated a DPU metabolite from tobacco tissue grown in the presence of ^{14}C-labelled DPU and identified this compound as a glucoside of DPU. This compound was inactive in the soybean callus assay and was 125 times less active than DPU itself in the tobacco callus bioassay. They concluded that this metabolite could not be responsible for the biological activity of DPU. Subsequently, Burrows (1976) examined the cytokinin-active constituents of tRNA from tobacco callus tissue which was grown on medium containing radiolabelled DPU as the sole source of cytokinin. Cis-io^{6}A and i^{6}A were the predominant cytokinins present in the tRNA and ms^{2}io^{6}A was a minor cytokinin-active component. Only a low level of ^{14}C was incorporated into the tRNA from DPU, and it was concluded that this incorporation resulted from ^{14}C released during the metabolism of DPU (probably as $^{14}CO_2$) and then reutilized by the callus and incorporated into ribonucleosides. On the basis of this result, it was concluded that if DPU (or its metabolites) is inducing cell division in tobacco callus by the same mechanism as the purine cytokinins, the mechanism of action of cytokinins is not related to the presence of cytokinins in specific tRNA species.

B. Naturally occurring cytokinins and their metabolites.

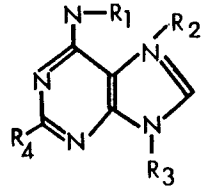
Aside from the original report by Shantz and Steward(1955) on the isolation of DPU from coconut milk, all other naturally occurring cytokinins identified to date have been purine derivatives. Zeatin,

the first such naturally occurring cytokinin to be identified, was isolated and partially identified from extracts of immature corn kernels by Miller (1961, 1962) and Letham (1963). Letham et al. (1964) established the structure of this compound as 6-(4-hydroxy-3-methyl-trans-2-butenylamino)purine. Since the discovery of zeatin, a number of other cytokinins have been isolated and identified from various organisms. With one exception (Hewett and Wareing, 1973; Horgan et al., 1973, 1975), all of the naturally occurring cytokinins for which structures have been determined are structurally related to N^6-(Δ^2-isopentenyl)adenine. Table 1 lists the structures of naturally occurring cytokinins.

Zeatin, its ribonucleoside, and ribonucleotide, have been isolated from many plant sources. These include immature corn kernel (Letham, 1966, 1968; Miller, 1967a), the mycorrhizal fungus, Rhizopogon roseolus (Miller, 1967b), apple fruitlets (Letham and Williams, 1969), cytokinin-independent soybean callus (Miura and Miller, 1969), rice roots (Yoshida and Oritani, 1971), buds and leaves of poplar, maple, and birch (Engelbrecht, 1971), grape vine canes (Skene, 1972), pea seedling roots (Short and Torrey, 1972), germinating lettuce seeds (Van Staden, 1973), buds and leaves of poplar (Hewett and Wareing, 1973a, 1973b), extracts of bleeding sap from sycamore (Horgan et al., 1973), various developing parts of cocklebur (Henson and Wareing, 1976) and white lupin (Davey and Van Staden, 1978a, 1978b, 1978c), and the female plant of Mercurialis ambigua (Dauphin et al., 1979).

The analog of zeatin bearing a saturated sidechain, dihydro-

Table 1. Structural formulae and names of the common naturally-occurring cytokinins



Structure				Chemical name	Common name
R_1	R_2	R_3	R_4		
$-CH_2CH=C<^{CH_3}_{CH_3}$	-H	-H	-H	N^6-(Δ^2-isopentenyl)adenine	Isopentenyladenine
$-CH_2CH=C<^{CH_3}_{CH_3}$	-H	-ribose	-H	N^6-(3-methyl-2-butenylamino)-9-ribofuranosylpurine N^6-(Δ^2-isopentenyl)adenosine	Isopentenyladenosine
$-CH_2CH=C<^{CH_3}_{CH_3}$	-H	-ribose	$-CH_2SH$	N^6-(3-methyl-2-butenylamino)-2-methylthio-9-ribofuranosyl purine	Methylthio-isopentenyl-adenosine
$-CH_2CH=C<^{CH_2OH}_{CH_3}$	-H	-H	-H	N^6-(4-hydroxy-3-methyl-2-butenyl)-adenine	trans-Zeatin
$-CH_2CH=C<^{CH_2OH}_{CH_3}$	-H	-ribose	-H	N^6-(4-hydroxy-3-methyl-2-butenyl-amino)-9-ribofuranosylpurine	trans-Ribosylzeatin

Table 1 continued.

$-CH_2CH=C<^{CH_2OH}_{CH_3}$	-H	-ribose	$-CH_2SH$	N^6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-ribofuranosyl purine	Methylthio-*trans*-ribosylzeatin
$-CH_2CH=C<^{CH_2OH}_{CH_3}$	-glucose	-H	-H	N^6-(4-hydroxy-3-methyl-2-butenylamino)-7-glucopyranosyl-purine	7-Glucosylzeatin (Raphanatin)
$-CH_2CH=C<^{CH_2OH}_{CH_3}$	-H	-glucose	-H	N^6-(4-hydroxy-3-methyl-2-butenylamino)-9-glucopyranosyl-purine	9-Glucosylzeatin
$-CH_2CH=C<^{CH_2O\text{-glucose}}_{CH_3}$	-H	-H	-H	N^6-(4-glucopyranosyloxy-3-methybuthylamino)purine	O-Glucosylzeatin
$-CH_2CH=C<^{CH_2OH}_{CH_3}$	-OH	-H	-H	N^6-(4-hydroxy-3-methyl-2-butenylamino)-2-hydroxy-purine	2-Hydroxyzeatin
$-CH_2CH_2CH<^{CH_2OH}_{CH_3}$	-H	-H	-H	N^6-(4-hydroxy-3-methyl-butyl-amino)purine	Dihydrozeatin
$-CH_2-C_6H_4(OH)$	-H	-ribose	-H	N^6-(O-hydroxybenzylamino)-9-ribofuranosyl purine	Hydroxybenzyladenosine

zeatin and/or its ribonucleoside, has been isolated from immature lupin seeds (Koshimitz et al., 1967), root, leaves, and seed of white lupin (Davey and Van Staden, 1978) and leaves of garden bean (Wang and Horgan, 1978), and pinto bean fruit (Krasnuk et al., 1971). N^6-(Δ^2-isopentenyl)adenine and its ribonucleoside have been reported to occur in an autonomous strain of tobacco tissue culture (Einset and Skoog, 1973; Dyson and Hall, 1972) as well as in the culture medium of Rhizopogon roseolus (Miura and Miller, 1969) and in the female plant of the Mercurialis ambigua (Dauphin et al., 1979). In contrast to other plant materials where only cytokinins bearing isoprenoid side chains have been found, the leaves of poplar have been reported to contain N^6-(O-hydroxy-benzylamino)adenosine (Hewett and Wareing, 1973; Horgan et al., 1973, 1975).

In addition to the free base, ribonucleoside, and ribonucleotide forms of cytokinins, plant tissues may also contain various types of cytokinin glucosides. The initial evidence for the existence of cytokinin glucosides came from studies of the metabolism of exogenously supplied, labeled cytokinins such as zeatin and benzyladenine (Delueze et al., 1972; Fox et al., 1973; Parker et al., 1972, 1973, 1975; Parker and Letham, 1974). However, several authors have reported that plant tissues contain endogenous cytokinin glucoside derivatives. Reports of this type include the 7-glucoside of zeatin from rice (Yoshida and Oritani, 1972) and the 9-glucoside of zeatin from bud and needles of conifer, Picea sitehensis (Lorenzi et al., 1975). Glucosides of zeatin and ribosylzeatin in which the glycosyl residue

is attached to the hydroxyl group of the isoprenoid side chain have been reported to occur in Vinca rosea crown gall tumor tissue (Peterson and Miller, 1977), and Wang et al. (1977) have reported the occurrence of high amounts of dihydrozeatin-O-ß-D-glucoside in leaves of Phaseolus vulgaris.

Recently, Parker et al. (1978) have reported that lupin seedlings contain a zeatin-amino acid conjugate called lupinic acid, identified as L-ß[4-hydroxy-3-methyl-trans-butenylamino)purine-O-yl]-alanine.

Cytokinin-active ribonucleosides also occur as constituents of tRNA molecules, where they are found adjacent to the 3' end of the anticodon in tRNA species that respond to codons beginning with U. In this form, cytokinins are widely distributed and occur in animal and microbial systems as well as in plants. The cytokinin-active ribonucleosides that occur in tRNA include i^6A, io^6A and their 2-methylthio derivatives, ms^2i^6A and ms^2io^6A (Skoog and Armstrong, 1970; Kende, 1971; Hall, 1973). Cytokinins are also produced in the free form by a number of microorganisms that act as plant symbionts and pathogens. In addition to the previously mentioned Rhizopogon roseolus, cytokinins have been identified in culture medium from Agrobacterium tumefaciens (Hahn et al., 1976; Kaiss-Chapman and Morris, 1977), Corynebacterium fascians (Scarbrough et al., 1973; Armstrong et al., 1976), and Rhizobium species (Phillips and Torrey, 1972).

C. Biosynthesis of free cytokinins

1. The biosynthetic pathway.

Until recently, evidence concerning the biosynthetic pathways involved in cytokinin production was based entirely on *in vivo* experiments using radioactively labelled adenine or adenosine. One of the eariest studies concerning cytokinin biosynthesis and metabolism was performed by Miller (1967) who analyzed the changes in cytokinin content in developing corn kernels after pollination. At early times following pollination, most of the extractable cytokinin activity was detected in the zeatin region of chromatograms. At later times, most of the activity was found in regions correspondingly to the ribonucleoside and ribonucleotide forms of zeatin. He concluded that zeatin was probably the first biosynthetic product in the pathway of cytokinin metabolism. Later, Miura and Miller (1969), supplied labelled adenine and hypoxanthine to *Rhizopogon roseolus* cultures. After four days growth on a shaker, radioactivity was found in ribosylzeatin released into the culture medium. Later, Miura and Hall (1973) incubated 2-day old cultures of *R. roseolus* for 6 days with radioactive adenosine and found a pronounced incorporation of label into i^6A and i^6Ade. They also demonstrated that this organism converted labelled i^6A to ribosylzeatin. These data suggested the pathway for the synthesis of zeatin (or ribosylzeatin) involved the attachment of the isopentenyl side chain to the N^6-amino group of adenine or adenosine followed by subsequent hydroxylation. Beutelmann (1973),

working with moss callus, observed a marked incorporation of labelled adenine into i^6Ade within eight hours after addition of the label to the cells. Peterson and Miller (1976) examined the biosynthesis of cytokinins in crown gall tumor tissue of Vinca rosea. In the presence of reduced nitrogen sources, they found that the tumor tissue rapidly absorbed labelled adenine from a liquid medium and within one hour incorporation into zeatin and ribosylzeatin could be detected. More recent work (Stuchbury et al., 1979) has demonstrated that the tumor tissue is able to convert labelled adenine into io^6A, io^6Ade and io^6AMP with maximum incorporation into these compounds occurring between 8 hours and 24 hours after the start of the incubation. These products then declined in amount, and there was an increase in the label associated with cytokinin glucosides. They suggested that AMP may be directly converted into zeatin ribonucleotide with subsequent conversion of the ribonucleotide into ribosylzeatin and zeatin. Einset and Skoog (1973) reported that a cytokinin autonomous strain of tobacco tissue converted ^{14}C-adenine into a compound with chromatographic properties similar to i^6Ade. Their methods did not permit the detection of incorporation into zeatin, although biological activity corresponding to zeatin, i^6Ade, and methylthiozeatin could be extracted from the tissue. Only very low levels of incorporation into i^6Ade could be detected in a cytokinin dependent line of the callus tissue. More recently Chen and Petschow (1978) isolated the cytokinins produced by cultured rootless tobacco plants in the presence of radioactive adenine, and identified these compounds as i^6Ade, i^6A, io^6A,

and io^{6}Ade. However, their results did not permit any conclusions concerning the biosynthetic sequence by which these compounds were produced.

Cytokinin biosynthesis in cell free systems has been achieved only recently. Taya et al. (1978) were the first to obtain such a system. The enzyme preparation was obtained from an unexpected source, the cellular slime mold, Dictyostelium discoideum. The Dictyostelium enzyme catalyzed the formation of i^{6}AMP from AMP and Δ^2-IPP. Chen and Melitz (1979) subsequently isolated the enzyme, Δ^2-IPP: AMP-Δ^2-isopentenyltransferase, from cytokinin-independent and -dependent tobacco callus tissue. Like the Dictyostelium enzyme, the tobacco enzyme catalyzed the biosynthesis of i^{6}AMP from Δ^2-IPP and 5'-AMP.

2. Transfer RNA as a possible source of free cytokinins.

The possibility that free cytokinins might be produced by degradation of cytokinin-containing tRNA molecules was originally suggested by Chen and Hall (1969). Several attempts have been made to determine whether turnover rates of tRNA are sufficient to liberate significant quantities of free cytokinins. Klämbt and coworkers examined this question using Lactobacillus acidophillus cultures (Leineweber and Klämbt, 1974) and primary roots of Zea mays (Klemen and Klämbt, 1974). They concluded that the turnover of tRNA molecules was sufficiently rapid to constitute a possibly significant source of free cytokinins in the cells of Zea mays. However, others have concluded that the turnover of tRNA molecules is not rapid enough to provide a source of significant quantities of free cytokinins (Teyssendier de la Serve

and Peaud-Lenoel, 1976). The results of such studies appear to be highly dependent upon the choice of organism and the technique used to determine tRNA half-life. Although, it appears conceivable that tRNA turnover is rapid enough to supply at least some of the free cytokinins produced in particular plant tissues, there is little evidence to support the concept that this is a significant source of free cytokinins.

Short and Torrey (1972) measured the amounts of free versus tRNA-bound cytokinins in pea roots. They concluded that free cytokinins were twenty-seven times more abundant than in tRNA and that either very rapid turnover rates and/or a slow breakdown of free cytokinins would be required for tRNA to account for the observed level of cytokinins in this tissue.

Evidence that tRNA degradation could not be the only pathway of cytokinin biosynthesis in plant tissues was obtained by Chen et al. (1974, 1976) using the labelled adenosine analogue 2-O-[1-(R)-(9-adenyl)-2-(hydroxy)ethyl]glycerol in which the 9-position is blocked. This compound was converted to the corresponding i^6A analogue by cytokinin-autotrophic tobacco tissue cultures, but little if any of label was incorporated into RNA in the tissue.

Burrows (1978) examined the cytokinin composition of tRNA from Lupinus luteus seeds and Populus robusta leaves. These tissues have been reported to contain dihydrozeatin and N^6-(2-hydroxybenzyl)adenosine, respectively, as their major free cytokinins (Koshimitz et al., 1967; Horgan et al., 1973, 1975). He was unable to detect either of

these cytokinins in the corresponding tRNA preparations. In these experiments, modification of zeatin to dihydrozeatin after its release from tRNA was not ruled out, but it appears unlikely that the hydroxybenzyl derivative found in Populus leaves could be produced by modification of cytokinins released from Populus tRNA as only N^6-isoprenoid derivatives were detected in the tRNA from this source.

Zeatin and its derivatives occur naturally in the form of both the cis and trans isomer. Generally, the trans isomer is found in the free forms in plant cells, and the cis forms occur in tRNA, but this rule is not absolute. Small amounts of the trans isomer have been reported in tRNA (Chapman et al., 1976; Vreman et al., 1972, 1974, 1978) and the cis isomer occurs free in the culture filtrates of some microorganisms (Armstrong et al., 1976; Scarbrough et al., 1973; Kaiss-Chapman and Morris, 1977), and has been isolated from potato tissue (Mauk and Langille, 1978). This raises the possibility that the free cis compounds may be derived from catabolism of tRNA. If so, the degradation of tRNA may be an important source of cytokinins in microbial systems and in some special cases in plant tissues. Further work will be needed to resolve this question.

3. The regulation of cytokinin metabolism.

The regulatory mechanisms controlling cytokinin levels in plant tissues are poorly understood. Certain genetic tumors occurring in hybrids of Nicotiana species (Smith, 1972) are presumably the result of derangements in the regulatory mechanisms controlling the biosyn-

thesis of cytokinins and other plant hormones. Tissue cultures derived from the tumorous hybrid (Nicotiana langsdorfii x Nicotiana glauca) as well as crown gall tumors induced by infection with Agrobacterium tumefaciens exhibit no requirement for exogenous cytokinins (Ahuja, 1962; Schaeffer et al, 1963; Smith, 1972; Lippincott and Lippincott, 1975).

Many normal plant tissues grow on defined tissue culture media that include an auxin and cytokinin. These tissues sometimes spontaneously lose their requirement for an exogenous source of auxin or cytokinin or both. Thereafter, the tissue can be propagated indefinitely without added auxin and/or cytokinin. This type of habituation phenomenon has been observed in cultures of a wide variety of plant species (Gautheret, 1955; Fox, 1963; Miura and Miller, 1969; Wood et al., 1969; Dyson and Hall, 1972; Einset and Skoog, 1973; Mok et al., 1980). These spontaneous transformations do not appear to involve mutational events as tobacco plants regenerated from single cell clones of cytokinin-independent cultures gave rise to tissue cultures that exhibited the normal cytokinin-dependent phenotype (Binns and Meins, 1973).

The auxin, 2,4-dichlorophenoxyacetic acid, has been reported to promote some cell division in cultures of cytokinin dependent soybean callus tissue (Witham, 1968; Fosket and Torrey, 1969) in the absence of an exogenous cytokinin. This suggests that auxin concentrations may exert some sort of regulatory influence on cytokinin biosynthesis.

Chemical agents that specifically affect cytokinin metabolism

are not yet available. However, a number of compounds containing reduced nitrogen have been reported to enhance cytokinin production in crown gall tumor tissue (Peterson and Miller, 1976) and in conditioned soybean root cells (Sargent and King, 1974). The mechanism of this effect is unknown. Treatment with streptomycin (Kaninek and Lustinec, 1974) or with morphactins or carcinogenic aminofluorenes has been reported to induce cytokinin autonomy in cytokinin-dependent tobacco tissue cultures (Bednar and Linsmaier-Bednar, 1971a, 1971b, 1971c; Bednar et al., 1973).

Ashton et al. (1979a, 1979b) isolated several different mutants of the moss Phycomitrella patens that appear to be defective in the regulation of either auxin or cytokinin metabolism, but the biochemical nature of the defects has yet to be determined. Durand and his coworkers (Durand, 1969; Dauphin et al., 1979; Kahlem et al., 1975) showed that male sterility in the dioecious plant Mercurialis appeared to involve altered cytokinin biosynthesis. High cytokinin levels appear to be associated with a shift toward female characteristics in the flowers.

It should be clear from the above discussion that a number of potentially valuable genetic materials as well as a number of interesting observations concerning chemical and genetic influences on cytokinin metabolism are available in the literature. However, little is known about the regulatory mechanism by which cytokinin metabolism is controlled in plant tissues.

III. MATERIALS AND METHODS

A. Materials

Chromatographic materials (Amberlite XAD-2, XAD-7, Sephadex LH-20) were obtained from Sigma Chemical Co. Porapak Q (150-200 Mesh) was purchased from Water Associates, Inc. Crude snake venom phosphodiesterase (Crotalus adamanteus), alkaline phosphatase (calf intestinal mucosa, type VII), ribonuclease T_1 (Aspergillus oryzae), cytokinin standards [ribosylzeatin, zeatin, N^6-(Δ^2-isopentenyl)adenine, N^6-(Δ^2-isopentenyl)adenosine, N^6-benzyladenosine, N^6-benzyladenine, and kinetin], N,N'-diphenylurea, 6-cyclohexylureidopurine, indole-3-acetic acid, 2,4-dichlorophenoxyacetic acid, and naphthalene acetic acid were also purchased from Sigma Chemical Co. Samples of 6-phenylureidopurine and 6-p-tolylureidopurine were kindly provided by Dr. F. Skoog (Institute of Plant Development, University of Wisconsin, Madison, Wisconsin). Picloram was a gift from Dow Chemical. The cytokinin, 6-cyclohexanemethylaminopurine was synthesized in this laboratory by S. Dixon. Adenine-8-^{14}C (specific activity, 62 mci/mmole) was obtained from Amersham Co. Omnifluor is a product of New England Nuclear. Wheat germ tRNA was previously isolated in this laboratory. Escherichia coli tRNA and brewer's yeast tRNA were purchased from Boehringer Mannheim.

Agrobacterium tumefaciens strain C58 was kindly provided by Dr. R. Morris (Department of Agricultural Chemistry, Oregon State University, Corvallis, Oregon). Seeds of Phaseolus lunatus L. cv.

Kingston were locally obtained in the greenhouse. Seeds of plant introduction lines (P.I. lines) of P. lunatus were provided by the Regional Plant Introduction Station, Washington State University, Pullman, Washington. Seeds of P. lunatus L. cv. Henderson Bush, Jackson Wonder, Nema Green were purchased from Asgrow Seed Company, Kalamazoo, Michigan.

B. Phaseolus tissue culture.

The Phaseolus tissue culture medium consisted of the mineral nutrients as described by Murashige and Skoog (1962) with the following organic substances added; sucrose (30 g/liter), myo-inositol (100 mg/liter), thiamine-HCl (1 mg/liter), nicotinic acid (5 mg/liter), and pyridoxine-HCl (0.5 mg/liter). Picloram (2.5 μM) was used to supply the auxin requirement of the Phaseolus callus tissue (Mok and Mok, 1977). Kinetin (5 μM) was included in medium used for callus initiation and stock cultures. The pH of the medium was adjusted to 5.7 and Difco Bacto-agar (10 g/liter) was added. The medium was dispensed into 125-ml Erlenmeyer flasks (50 ml per flask) and autoclaved at 120°C for 15 min. In tests of the effect of DPU on callus growth, appropriate amounts of DPU were dissolved in dimethylsulfoxide (Schmitz and Skoog, 1970) and added to the autoclaved tissue culture flasks prior to solidification of the medium. The final amount of dimethyl sulfoxide in the tissue culture medium was 0.025 ml per flask. Ureidopurines were also added to the tissue culture medium after autoclaving using the same technique.

Seeds of each genotype were surface sterilized with 2% sodium-hypochlorite and germinated under sterile condition (Mok and Mok, 1977). Hypocotyl tissue of 5-day-old seedlings was cut into slices and three discs (1 mm thick) were planted in each flask.

The callus tissue that formed on the initial explants was transferred once (first passage) on the same medium used for callus initiation. Tests for DPU response were performed in the second passage of the callus tissue by using 3-week-old first passage cultures as stock tissue. Three pieces of callus weighing approximately 25 mg each were planted per flask. Tissues were harvested and weighed after 35 days of growth at 28°C in the dark. All tests were repeated at least once by using newly established cultures.

C. Tobacco tissue culture

Cytokinin-dependent tobacco pith callus (Nicotiana tabacum L., var. Wisconsin 38) was grown on RM-1965 medium (Linsmaier and Skoog, 1965) containing indole-3-acetic acid (2 mg/liter) and 0.14 μM kinetin. Cytokinin-autonomous tobacco callus,derived from cytokinin-dependent tobacco tissue (Einset and Skoog, 1973) was maintained on cytokinin-free medium containing 2 mg/liter indole-3-acetic acid. In experiments involving DPU, appropriate amounts of DPU were dissolved in dimethyl sulfoxide and added to the autoclaved medium prior to solidification of the medium. The final amount of dimethyl sulfoxide in the tobacco tissue culture medium was 0.050 ml per flask. Other culture conditions were the same as for Phaseolus.

D. Culture of Agrobacterium tumefaciens

Agrobacterium tumefaciens C58 was kindly provided by Dr. R. Morris (Department of Agricultural Chemistry, Oregon State University, Corvallis, Oregon). The cells were cultured on a defined medium as described by Chapman and Morris (1976). The composition of culture medium is shown in Table 2. Growth of cells in medium was accomplished in Fernbach flasks which were agitated on a rotary shaker at 150 cycles/minutes at a 26°C. The cells were separated from the medium by centrifugation at 14,500 g for 25 minutes. The cell-free supernatant was collected in silane-treated glassware and used as the culture filtrate for further purification of cytokinins.

E. Methodology for detection of in vivo cytokinin biosynthesis from biological materials.

1. Trace enrichment of synthetic cytokinins on Amberlite XAD-2 columns.

Amberlite XAD-2 (Sigma Chemical Co.) was prepared for use by washing the resin in distilled water at least five times and packing it into the column. The packed columns were extensively washed with distilled water, 70% ethanol, and distilled water before use. Chromatographic samples were adjusted to pH 5.8 prior to loading on the column. Following sample application, the columns were immediately washed with 30 bed volumes of distilled water. The adsorbed cytokinins were eluted with 70% ethanol. The first five bed volumes and the second five bed

Table 2. Medium for Agrobacterium tumefaciens C58

Component	g/liter	mg/liter
Sucrose	15	
$(NH_4)_2SO_4$	2	
KH_2PO_4 *	0.2	
K_2HPO_4 *	0.8	
Biotin**		1
KNO_3	0.4	
$MgSO_4$ $7H_2O$	0.2	
$CaCl_2$ $2H_2O$ ***	0.2	
$FeCl_3$ $6H_2O$	0.01	
Na_2MoO_4 $2H_2O$		0.2
$ZnSO_4$ $7H_2O$		0.2
H_3BO_4		0.2
$MnSO_4$ H_2O		0.2
$CuSO_4$ $5H_2O$		0.015
$CoCl_2$ $6H_2O$		0.001

*KH_2PO_4 and K_2HPO_4 were separately dissolved.

**Biotin was dissolved by heating in appropriate amount of distilled water and added into the medium.

***$CaCl_2$was separately dissolved.

volumes of eluate were collected separately. Finally the columns were eluted with five bed volumes of 95% ethanol. Each sample was taken to dryness under reduced pressure at 38°C.

2. Isolation of cytokinins from tRNA preparations.

Each tRNA sample (600 A_{260} units) was hydrolyzed enzymatically as described by Hall (1964). The tRNA was dissolved in glass distilled water (20 A_{260} units per ml) and the pH of the solution was adjusted to 7.5. Ribonuclease T_1 (Aspergillus oryzae, Sigma Chemical Co.) (5 units per A_{260} unit tRNA) was added, and the solution was incubated at 35°C for 2 hours. The pH was adjusted as needed during the incubation period. At the end of the two hours incubation with RNase T_1, the pH of the solution was adjusted to 8.6 and the following were added: 0.1 M $MgSO_4$ (0.01 ml/ml incubation volume), crude snake venom phosphodiesterase (Crotalus adamanteus, Sigma Chemical Co.) (0.01 mg/A_{260} unit), alkaline phosphatase (calf intestinal mucosa, Type VII, Sigma Chemical Co.) (0.01 units/A_{260} unit). Drops of toluene were added periodically to prevent bacterial growth. After eight hours of incubation, a second batch of 0.1 M $MgSO_4$ (0.005 ml/ml), crude snake venom (0.003 mg/A_{260} unit), and alkaline phosphatase (0.01 units/A_{260} unit) were added. Incubation was continued for another 16 hours. The hydrolysis was terminated by adjusting the pH to 5.8 and by heating the hydrolysate at 60°C for 10 min.

The tRNA hydrolysates were loaded on the Amberlite XAD-2 columns (0.9 x 30 cm) equilibrated with distilled water as previously described. Following sample application, the columns were subsequently washed with

30 bed volumes of distilled water. The adsorbed cytokinins were eluted with 70% ethanol. The first five bed volumes and the second five bed volumes of eluate were collected separately. Finally the columns were eluted with five bed volumes of 95% ethanol. Each sample was taken to dryness *in vacuo* at 38°C.

3. Trace enrichment of cytokinins in a culture filtrate of *A. tumefaciens* C58.

The culture filtrate(4,700 ml) of *A. tumefaciens* C58 was applied to a 2.5 x 50 cm column of Amberlite XAD-2 prepared as described above. The column was then washed with 30 bed volumes of distilled water. Bound material was eluted with 70% ethanol. The first five bed volumes and the second five bed volumes of eluate were collected separately. Each step sample was bioassayed for cytokinin activity. The 70% ethanol eluate was taken to dryness and dissolved in 4.7 ml of 33% ethanol and chromatographed on a Sephadex LH-20 column (2 x 57 cm) in the same solvent (Armstrong *et al.*, 1969) and the eluate was collected in 9 ml fractions. After elution was completed, synthetic standards; zeatin, ribosyl-zeatin, N^6-isopentenyladenosine, and N^6-isopentenyladenine were applied to the same column. The fractions were bioassayed after pooling them into groups based the standard eluation profiles.

4. Partial purification of cytokinins from *Phaseolus* callus.

A total of 4,000 g of cytokinin-autonomous callus tissue derived from *P. lunatus* cv. Kingston was harvested after 26 days growth and homogenized in a Waring Blender in 2½ volumes of cold 95% ethanol.

The homogenate was immediately filtered through Whatman No. 42 filter paper. The combined filtrates from the 4,000 g of tissue (pH 5.8) were passed through a 2.5 x 30 cm column of Amberlite XAD-2 which was previously equilibrated with 70% ethanol. The column was washed with an additional ten bed volumes of 70% ethanol, and total eluates reduced to an aqueous solution by evaporation under reduced pressure at 38°C. The aqueous solution was diluted to the original volume of the 70% ethanol extract with glass distilled water. This aqueous solution was passed through a 2.5 x 30 cm column of Amberlite XAD-2 equilibrated with distilled water. The column was then washed with 30 bed volumes of distilled water followed by ten bed volumes of 70% ethanol. The water wash was discarded and the ethanol eluate collected and evaporated to dryness as described previously. The solids recovered from the 70% ethanol eluate were extracted six times using 50 ml of the upper phase of ethyl acetate-water (5:1, v/v) for each extraction. The combined ethyl acetate extracts were reduced to dryness, redissolved in 20 ml of 33% ethanol, and centrifuged at 10,000 g for 25 minutes. The supernatant was evaporated to dryness, and redissolved in 5 ml of 33% ethanol for chromatography on Sephadex LH-20. The solids remaining after ethyl acetate extraction were taken to dryness, and reextracted six times with ethyl acetate: t-butanol (2:1, v/v) (50 ml per extraction). The extract was taken to dryness and redissolved in 5 ml of 33% ethanol for chromatography on Sephadex LH-20.

5. Metabolism of ^{14}C-adenine in tobacco tissue

For attempts to detect cytokinin biosynthesis from ^{14}C-adenine, cytokinin-autonomous tobacco callus tissue was selected from 25-day-old cultures. At this time, the fresh weight of the tissue was approximately 10 g per flask. One-ml of distilled water containing 2 μCi of 8-^{14}C-adenine (62 mc/mmole, Amersham) was aseptically dropped on the surface of tissue. The tissue was incubated at 27°C in the darkness for 5 hours. Cytokinins were extracted with 95% cold ethanol (25 ml/10 g), and cell debris was removed by successive filtration through Whatman 42 filter paper and Millipore filters. The ethanol extract was evaporated under reduced pressure at 38°C and redissolved in 2 ml of 33% ethanol for direct loading on Sephadex LH-20 or dissolved in 50 ml of distilled water for purification on Amberlite XAD-2. The latter aqueous solution was loaded on an Amberlite XAD-2 column (0.9 x 30 cm) prepared as before. The column was washed with distilled water (30 bed volumes) and then eluted with ten bed volumes of 70% ethanol. The ethanol eluate was evaporated to dryness and dissolved in 2 ml of 33% ethanol. This sample was chromatographed on a Sephadex LH-20 column (1.5 x 50 cm) in 33% ethanol. A total of 6 bed volumes was collected in 8 ml fractions at a flow rate of 24 ml/hour. Half volume of each column fraction was evaporated to dryness in scintillation vials. The samples were taken up in 5 ml of the toluene-based Omnifluor scintillation liquid and counted on a Packard Tri Carb liquid Scintillation spectrometer, Model 3385. The remainder of the radioactive fractions which eluted out at the same position as the cytokinin standards, io^{6}A, io^{6}Ade, i^{6}A,

and i^6Ade, were pooled and dried under reduced pressure at 35°C. i^6A and i^6Ade pools were dissolved in 2 ml of water with 0.2 mg of authentic unlabeled i^6A and i^6Ade and chromatographed on a Sephadex LH-20 column (1.5 x 52 cm) in distilled water. Each fraction was dried in a scintillation vial and assayed for ^{14}C in 5 ml of the toluene-based Omnifluor scintillation liquid.

The io^6A and io^6Ade pools were dissolved in one ml of 24% ethanol with authentic unlabelled io^6A and io^6Ade, respectively, and chromatographed on a Porapak Q (Water Associates, Inc., 150-200 Mesh) column in 24% ethanol (Armstrong and Skoog, 1975). The Porapak Q column was prepared by suspending five grams of Porapak Q in 24% ethanol for 5 hours. The resin was rinsed several times with 24% ethanol and packed into a 0.9 x 30 cm column. The column was equilibrated for two days with 24% ethanol. Each sample was loaded and eluted with 24% ethanol, and fractions were collected at a flow rate of about 10 ml/hour. Each fraction was dried in a scintillation vial, and counted for ^{14}C.

F. Tobacco callus bioassay for cytokinin activity

Samples from tRNA preparations were acid hydrolyzed in 5 ml 0.1 N HCl (100°C, 45 min.) prior to bioassay. The hydrolysates were rapidly cooled and neutralized with 0.1 N NaOH before incorporation into 100 ml of bioassay medium.

Bioassay samples from A. tumefaciens and P. lunatus callus tissue preparations were dissolved in 20 ml of distilled water by boiling in a water bath for 45 minutes and incorporated into 100 ml of RM-1965 medium (Linsmaier and Skoog, 1965). All bioassay samples were tested

in five-fold serial dilution in the tobacco callus bioassay. Four replicate 50 ml Erlenmeyer flasks, each containing 20 ml of medium, were prepared for each concentration tested. The flasks were sterilized by autoclaving. The four replicates of each dilution were planted with three pieces (approximately 20 mg each) of kinetin-dependent Nicotiana tabacum L. cv. Wisconsin 38 callus tissue. Standards, medium containing 0 to 16 µg/liter kinetin, were planted with each assay. The tissue was grown at 28°C in darkness and the callus was harvested after 35 days. The cytokinin activity of the test samples was determined comparing fresh weight yields with a standard curve prepared from the weights of the kinetin standards. Cytokinin activity was expressed as kinetin equivalents (KE), defined the µg of kinetin required to give the same activity as the test sample under the specified bioassay conditions.

IV. RESULTS

A. Response of Phaseolus callus tissues to N,N'-diphenylurea.

1. The activity of N,N'-diphenylurea in promoting P. lunatus callus growth.

The cytokinin activity of DPU in promoting P. lunatus callus growth was first tested in cultures derived from hypocotyl tissues of P. lunatus cv. Kingston and P.I. 257422. The results of this initial test are illustrated in Figure 1 and Table 3. As shown in Figure 1, response to DPU of P. lunatus cv. Kingston callus was detectable at concentration of 32 and 100 μM DPU. Although the amount of growth was irregular, the stimulation of callus growth by DPU was repeatably observed in tissues derived from different seeds of this cultivar. It should be noted that the four replicate flasks shown in Figure 1 represent callus tissue derived from four different seeds. Comparable results were obtained in repeated tests with newly established cultures. The variability in the growth of P. lunatus cv. Kingston callus tissue on DPU-containing medium contrasted with uniform and rapid growth of this genotype on medium containing kinetin (or other cytokinin-active adenine derivatives).

The presence of genetic variations in P. lunatus P.I. 257422 was evidenced by differences in seed coat color (red vs. white seeds) and callus tissues derived from the different seed types exhibited differences in their response to DPU (Table 3). Callus tissues derived from seedlings of red seed origin exhibited a weak response to DPU,

Figure 1. Representative callus cultures of P. lunatus cv. Kingston on DPU-containing medium and control medium.

Individual flasks of a treatment represent callus derived from different seeds. The average fresh weights (g per flask) of 16 flasks are given at the right of the figure.

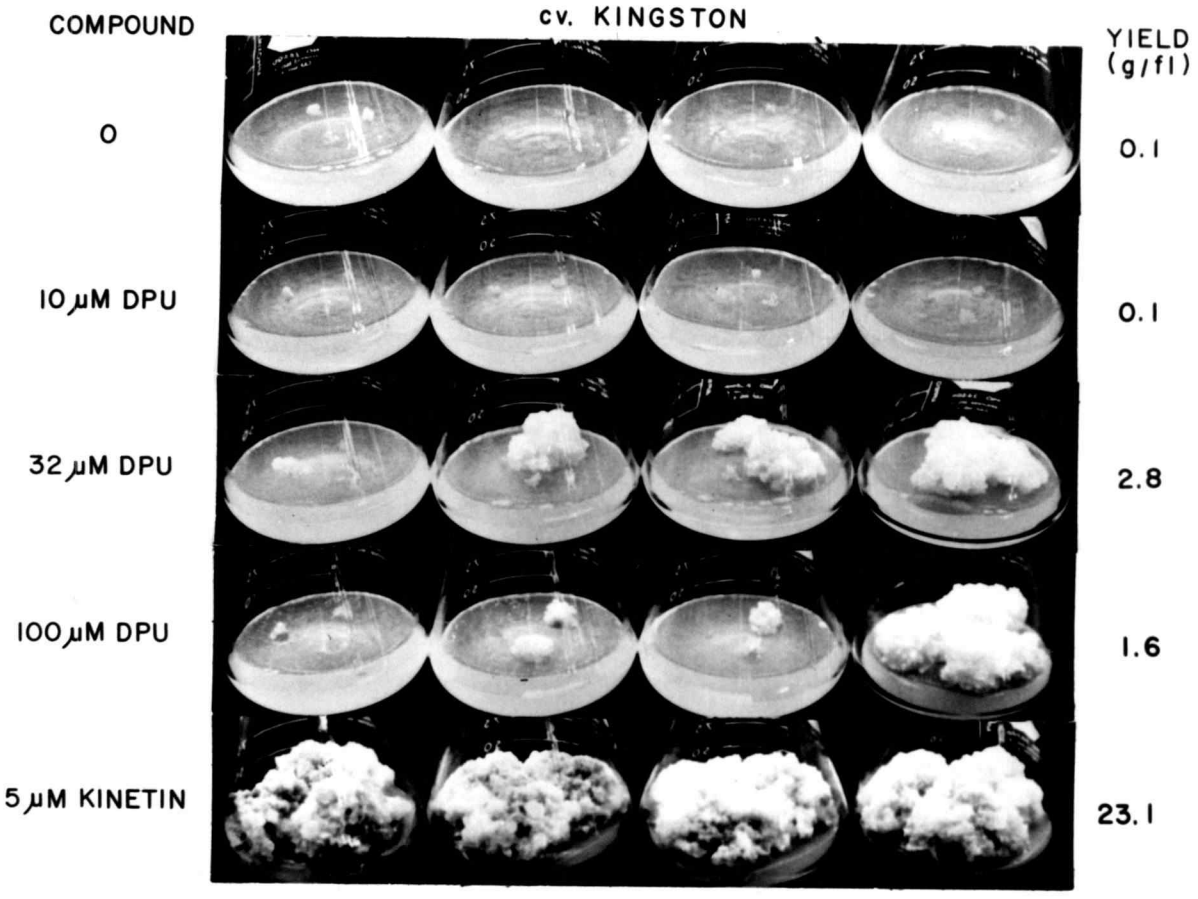

COMPOUND
cv. KINGSTON
YIELD (g/fl)
0
0.1
10 µM DPU
0.1
32 µM DPU
2.8
100 µM DPU
1.6
5 µM KINETIN
23.1

Table 3. Effect of N,N'-diphenylurea on growth of callus cultures derived from Phaseolus lunatus P.I. 257422

DPU concentration (μM)	Fresh weight, g per flask*	
	White seed origin	Red seed origin
0	0.87	0.08
0.1	0.47	0.10
0.3	1.69	0.10
1	0.60	0.25
3	3.15	0.13
10	6.92	0.51
32	6.59	1.32
100	3.14	1.01
5 μM Kinetin	22.24	15.54

*Average of eight flasks

but those of white seed origin responded vigorously and uniformly at concentrations of DPU from 10 to 100 μM. Response to DPU could be detected at concentrations as low as 0.3 μM, but growth was irregular at the lower concentrations.

2. Induction of cytokinin-autonomy by N,N'-diphenylurea.

The irregular response of P. lunatus callus tissues to DPU suggested that the activity of DPU in promoting the growth of P. lunatus callus tissue might be associated with an induction of cytokinin autonomy (i.e., a promotion of endogenous cytokinin biosynthesis in the callus tissue). To test this hypothesis, callus tissues from cv. Kingston and P.I. 257422 (w) previously grown on DPU-containing medium and control cultures maintained on 5 μM kinetin were transferred to cytokinin-free medium. The results of this test are shown in Figure 2 and Table 4. Callus tissue previously cultured on DPU-containing medium had acquired the capacity for continued growth in the absence of cytokinin, whereas callus tissue maintained on kinetin-containing medium did not exhibit any growth (cv. Kingston) or only slight growth (P.I. 257422-w) on the cytokinin-free medium. After one passage on DPU-containing medium, callus growth was somewhat irregular on cytokinin-free medium, but after two or three passages on medium containing 32 or 100 μM DPU, cultures grew vigorously and uniformly in the absence of cytokinin. Some of these callus tissues have been maintained on cytokinin-free medium for over a year (over 12 transfers) with no loss of vigor.

Figure 2. Representative callus cultures of P. lunatus cv. Kingston on cytokinin-free medium after one passage on DPU-containing medium or control medium.

Individual flasks represent callus derived from different seeds. The average fresh weights (g per flask) of 32 flasks are given at the right of the figure.

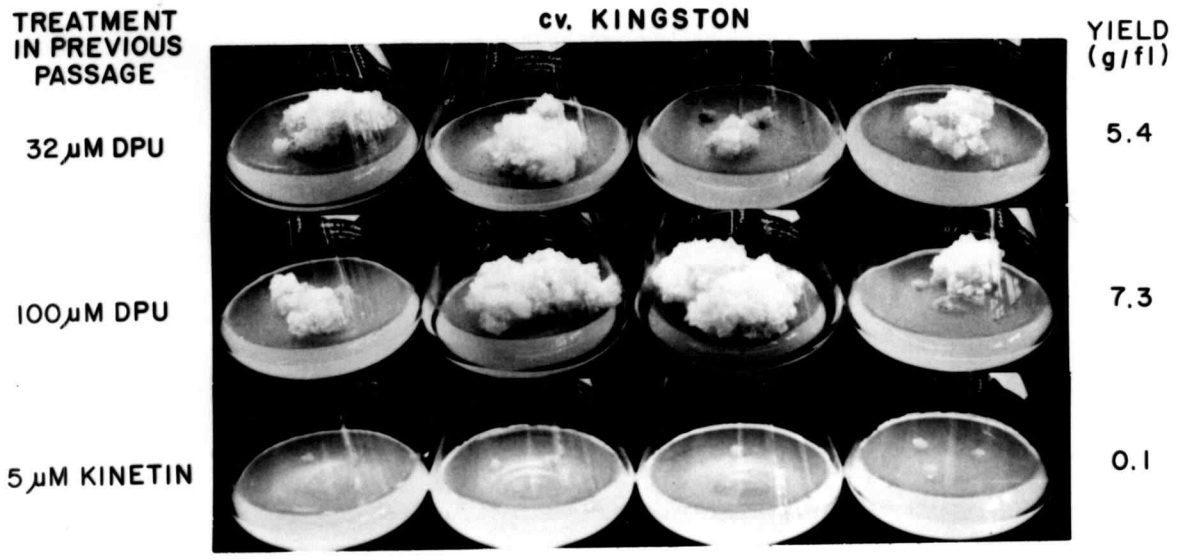

TREATMENT IN PREVIOUS PASSAGE
cv. KINGSTON
YIELD (g/fl)
32 µM DPU
5.4
100 µM DPU
7.3
5 µM KINETIN
0.1

Table 4. Effect of N,N'-diphenylurea in inducing cytokinin autonomy in callus cultures of Phaseolus lunatus P.I. 257422 (W)

Treatment in previous passage	Fresh weight (g per flask) on cytokinin-free medium*
3 µM DPU	5.70
10 µM DPU	6.51
32 µM DPU	5.17
100 µM DPU	3.14
5 µM Kinetin	0.32

*Average of eight flasks

3. Determination of optimal concentrations for picloram and kinetin in tissue cultures of seven P. lunatus genotypes.

To determine optimal conditions for establishing callus cultures of different P. lunatus genotypes, hypocotyl tissues of seven P. lunatus genotypes were explanted on the medium-containing a range of picloram concentrations and 5 μM kinetin as cytokinin source. Most of the callus tissues derived from the seven genotypes grew well on the 1.25, 2.5 and 5 μM concentrations of picloram. After 21 days in culture, the tissues were transferred to fresh media containing three different concentrations of kinetin and the picloram concentrations indicated above. The results are shown in Table 5. Although some differences in the response of different genotypes were evident at the lower auxin and cytokinin concentrations, all tissues grew well on 2.5 μM picloram and 5 μM kinetin. Auxin and cytokinin concentrations higher than this gave some increase in yield, but the tissues exhibited browning at a relatively early stage in the culture period. The quality of tissues cultured on the medium containing 2.5 μM picloram and 5 μM kinetin was best in most genotypes.

4. Genetic variation in the response of P. lunatus callus tissues to N,N'-diphenylurea.

A number of P. lunatus genotypes have now been tested for their ability to respond to DPU in tissue culture. Callus cultures of the various P. lunatus genotypes were established from hypocotyl explants placed on a medium containing 2.5 μM picloram and 5 μM kinetin. The

Table 5. Effect of picloram and kinetin on callus tissue growths of seven Phaseolus lunatus genotypes

Picloram (μM)	Kinetin (μM)	Genotype						
		202830	256845	257409	257422(W)	257560	260415	264239
		Fresh weight (g/ flask)						
	2.5	14.09	5.08	16.46	15.69	15.07	6.42	10.29
1.25	5.0	16.36	8.28	20.38	16.38	14.45	9.54	9.95
	10.0	16.72	10.48	21.96	17.84	19.44	12.45	13.03
	2.5	13.63	5.70	14.24	15.72	16.97	10.66	11.23
2.50	5.0	15.50	11.26	20.17	16.85	16.73	13.26	11.83
	10.0	16.10	13.15	22.84	20.16	20.08	15.25	15.14
	2.5	7.80	5.47	10.29	14.90	15.78	12.02	10.24
5.00	5.0	11.65	11.62	15.98	16.25	18.52	15.44	11.09
	10.0	10.27	12.37	16.09	17.45	19.24	18.77	14.15

Slices of hypocotyl tissue of 5-day-old seedlings were explanted on the medium containing six different concentrations of picloram (0.3, 0.6, 1.25, 2.5, 5, and 10 μM) and 5 μM kinetin, and induced callus tissues. Callus tissues were tested for determination of optimal concentrations. The average fresh callus weight of 4 flasks was determined.

callus tissue that formed on the explants was transferred once on the same medium and tested for ability to respond to DPU by transfer to a medium containing 2.5 μM picloram and 32 μM DPU. Control cultures were maintained on the kinetin containing media. The genotypes tested could be classified into three groups on the basis of the response of the corresponding callus tissues to DPU. The most vigorous growth on DPU-containing media was observed with callus tissues derived from P. lunatus P.I. 264239, 257409, cv. Henderson Bush, and cv. Jackson Wonder (Figure 3). Intermediate growth was observed with P. lunatus genotypes P.I. 257560, 180324, 256812, and cv. Nema Green (Figure 4). The growth of callus tissues derived from these genotypes was irregular and similar to that of P. lunatus cv. Kingston. No growth or very limited growth on DPU-containing medium was observed with callus tissues derived from P. lunatus genotypes P.I. 202830, 260415, 256845, and 257547 (Figure 5).

The ability of DPU to induce cytokinin-autonomous growth in the P. lunatus callus tissues was tested by transferring the tissues grown on DPU-containing medium and control cultures maintained on 5 μM kinetin to cytokinin-free medium. The results of these tests are shown in Figures 6 and 7. Callus tissues previously cultured on DPU-containing medium exhibited continued growth on the cytokinin-free medium. DPU-grown tissues derived from some genotypes such as P.I. 180324, 256812 and cv. Jackson Wonder, grew vigorously and uniformly on cytokinin-free medium. Generally, callus tissues grown on the kinetin-containing medium exhibited little if any growth when

Figure 3. Effect of N,N'-diphenylurea in promoting callus growth of four P. lunatus genotypes cv. Henderson Bush, Jackson-Wonder, P.I. 257409, and P.I. 264239.

Callus tissues of P. lunatus genotypes cv. Henderson Bush, Jackson Wonder, P.I. 257409, and P.I. 264239 were tested for ability to respond to DPU by transfer to a medium containing 2.5 μM picloram and 32 μM DPU. Control cultures were maintained on 2.5 μM picloram and 5 μM kinetin. The average fresh weights (g per flask) of eight flasks are given at the right of figure. Individual flasks represent callus derived from different seeds.

CV. HENDERSON BUSH

CV. JACKSON WONDER

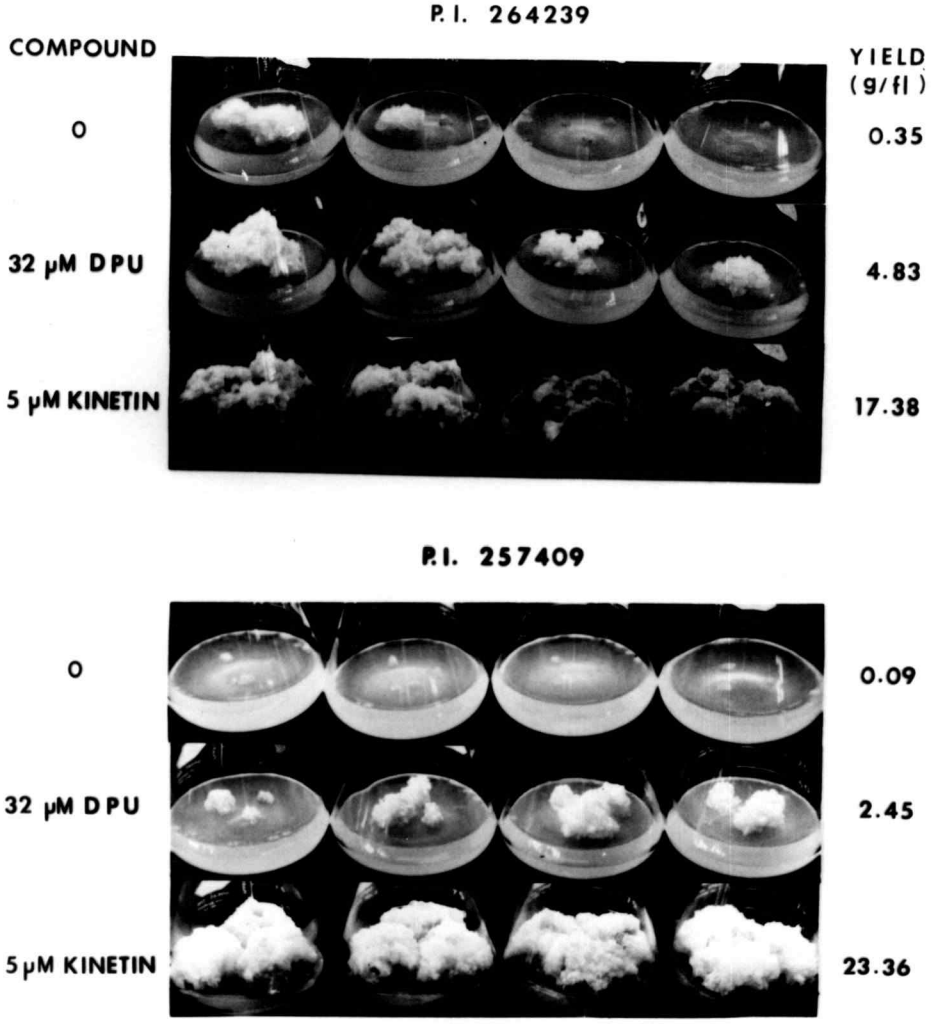
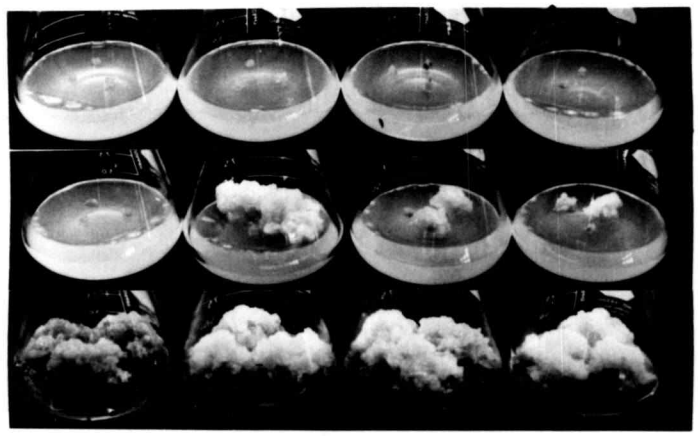

P. I. 264239
COMPOUND
YIELD (g/fl)
0
0.35
32 μM DPU
4.83
5 μM KINETIN
17.38
P.I. 257409
0
0.09
32 μM DPU
2.45
5 μM KINETIN
23.36

Figure 4. Effect of N,N'-diphenylurea in promoting callus growth of four P. lunatus genotypes P.I. 257560, 180324, 256812, and cv. Nema Green.

Callus tissues of P. lunatus genotypes P.I. 257560, 180324, 256812, and cv. Nema Green were tested for ability to respond to DPU by transfer to a medium containing 2.5 μM picloram and 32μM DPU. Control cultures were maintained on 2.5 μM picloram and 5 μM kinetin. Individual flasks represent callus derived from different seeds. The average fresh weights (g/flask) of eight replicate flasks are given at the right of the figure.

P. I. 257560



P. I. 180324

P.I. 256812

CV. NEMA GREEN

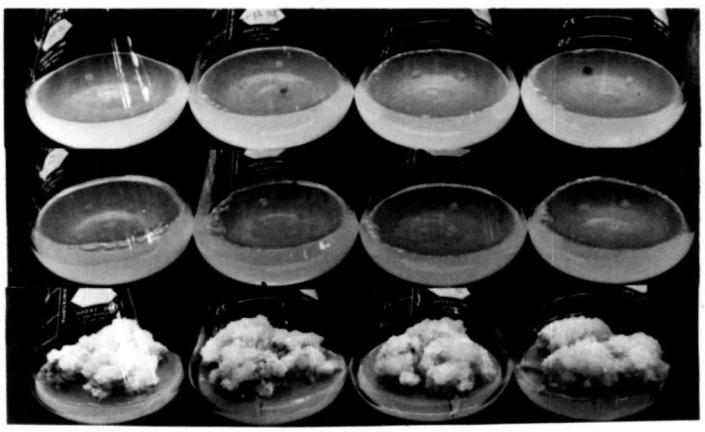
Figure 5. Effect of N,N'-diphenylurea in promoting callus growth of four P. lunatus P.I. 202830, 260415, 256845, and P.I.257547.

The callus tissues of P. lunatus genotypes P.I. 202830, 260415, 256845, and P.I. 257547 were tested for ability to respond to DPU by transfer to a medium containing 2.5 μM picloram and 32 μM DPU. Control cultures were maintained on 2.5 μM picloram and 5 μM kinetin. Individual flasks represent callus derived from different seeds. The average fresh weights (g per flask) of eight flasks are given at the right of figure.

P.I. 202830

COMPOUND	YIELD (g/fl)
0	0.05
32 μM DPU	0.08
5 μM KINETIN	14.75

P.I. 260415



0	0.06
32 μM DPU	0.06
5 μM KINETIN	15.66

P. I. 256845

P. I. 257547

Figure 6. Effect of N,N'-diphenylurea in inducing cytokinin-autonomy in callus tissues derived from four P. lunatus genotypes cv. Henderson Bush, Jackson Wonder, P.I. 257409, and P.I. 264239.

After one passage on 32 μM DPU-containing medium or 5 μM kinetin-containing medium, the callus tissues were transferred to cytokinin-free medium. Individual flasks represent callus derived from different seeds. After a culture period of five weeks on cytokinin-free medium, the callus tissues were harvested and the average fresh weights (g/flask) of 8 flasks are given at the right of the figure.

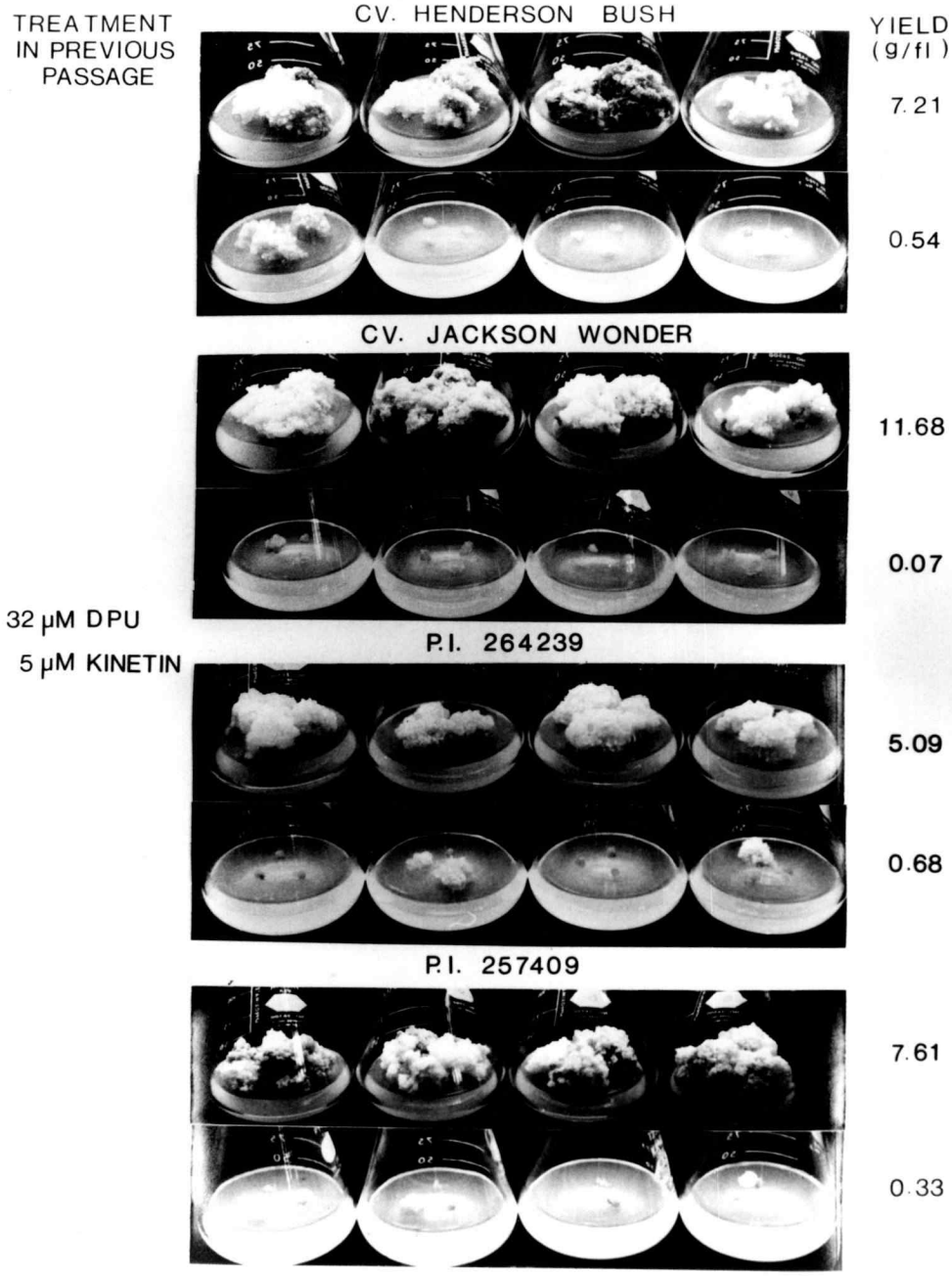
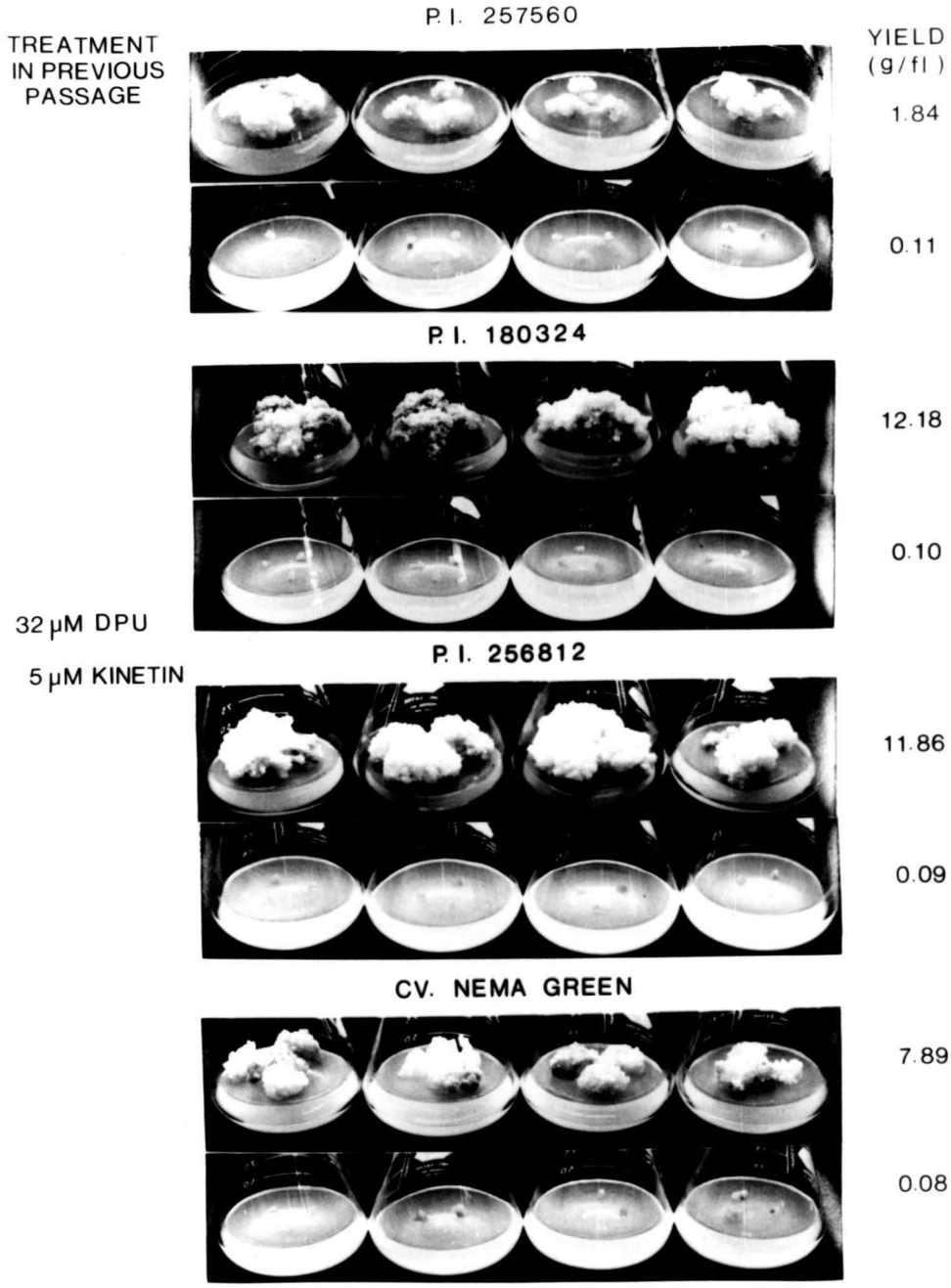

TREATMENT IN PREVIOUS PASSAGE
CV. HENDERSON BUSH
YIELD (g/fl)
7.21
0.54
CV. JACKSON WONDER
11.68
0.07
32 μM DPU
5 μM KINETIN
P.I. 264239
5.09
0.68
P.I. 257409
7.61
0.33

Figure 7. Effect of N,N'-diphenylurea in inducing cytokinin-autonomy in callus tissues derived from four P. lunatus genotypes P.I. 180324, 256812, 257560, and cv. Nema Green.

After one passage on 32 μM DPU-containing medium or 5 μM kinetin-containing medium, the callus tissues were transferred to cytokinin-free medium. Individual flasks represent callus derived from different seeds. After a culture period of five weeks on cytokinin-free medium, the callus tissues were harvested and the average fresh weight (g/flask) of 8 flasks are given at the right of the figure.


P. I. 257560
TREATMENT IN PREVIOUS PASSAGE
YIELD (g/fl)
1.84
0.11
P. I. 180324
12.18
0.10
32 µM DPU
5 µM KINETIN
P. I. 256812
11.86
0.09
CV. NEMA GREEN
7.89
0.08

transferred to cytokinin-free medium,although some genotypes did give rise to occasional cytokinin-autonomous callus pieces under these conditions. Genotypes such as P.I. 260415, which did not grow on the DPU-containing medium, exhibited little if any tendency for cytokinin-autonomous growth when transferred from kinetin-containing media to cytokinin-free media.

5. Interaction between kinetin and N,N'-diphenylurea in inducing cytokinin autonomy in Phaseolus tissue culture.

To test for possible interactions between kinetin and DPU in the induction of cytokinin-autonomous growth, three P. lunatus genotypes, cv. Jackson Wonder, P.I. 257422 (w) and P.I. 260415, were grown on media containing various concentrations of kinetin in the presence and absence of DPU. The results are shown in Figure 8. Interestingly, the presence of DPU in the medium did not change the kinetin concentration required for optimum growth in any of the genotypes tested. Genotype P.I. 260415 did not exhibit any response to DPU in either the presence or absence of kinetin. This genotype also required much higher concentrations of kinetin than the other two genotypes.

To test for cytokinin-autonomous growth, the callus cultures derived from the three genotypes and grown on media containing kinetin, DPU, or kinetin + DPU were transferred to cytokinin-free medium. The results were shown in Table 6. Unexpectedly, the cultures derived from Jackson Wonder and P.I. 257422 (w) and grown on less than optimal concentrations of kinetin alone in the previous passage exhibited

Figure 8. Interactions between kinetin and N,N'-diphenylurea in promoting callus growth of Phaseolus lunatus genotypes.

Callus tissues of P. lunatus cv. Jackson Wonder (A), P.I. 257422-w (B), P.I. 260415 (C) were planted on the medium containing 2.5 μM picloram and various concentrations of kinetin (o-----o), DPU (★———★), and a range of kinetin + 30 μM DPU (●———●). The tissues were harvested and weighed after 35 days. The average fresh callus weight of the four replicate flasks was determined.

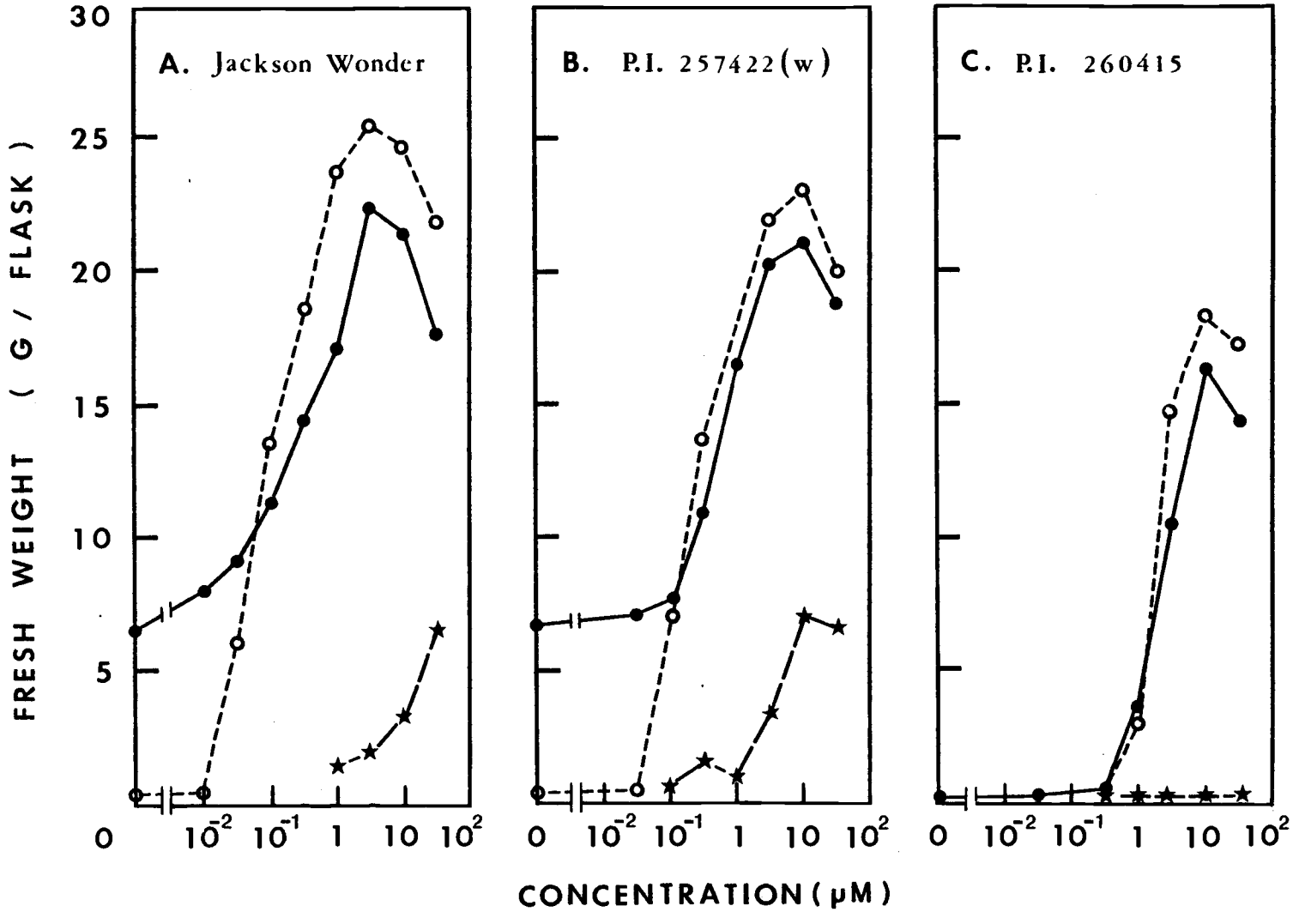

A. Jackson Wonder
B. P.I. 257422(w)
C. P.I. 260415
FRESH WEIGHT (G / FLASK)
30
25
20
15
10
5
0
0
10^{-2}
10^{-1}
1
10
10^{2}
CONCENTRATION (μM)

Table 6. Interaction of kinetin and N,N'-diphenylurea in inducing cytokinin autonomy in callus cultures of Phaseolus lunatus P.I. 260415, 257422(w), and cv. Jackson Wonder.

Treatment in previous passage	Fresh weight (g/flask)*		
	260415	257422(w)	Jackson Wonder
0	0.06	0.35	0.10
0.03 μM K	—**	—	7.81
0.1 μM K	—	5.86	1.75
0.3 μM K	—	0.54	0.68
1 μM K	0.06	0.64	0.10
3 μM k	0.06	0.20	0.07
10 μM K	0.07	0.31	0.11
32 μM K	0.18	0.32	1.14
32 μM DPU	0.05	8.64	10.21
0.03 μM K + 32 μM DPU	—	4.70	9.92
0.1 μM K + 32 μM DPU	—	5.46	9.19
0.3 μM K + 32 μM DPU	—	2.58	2.28
1 μM K + 32 μM DPU	0.23	1.29	4.76
3 μM K + 32 μM DPU	0.06	0.52	0.26
10 μM K + 32 μM DPU	0.11	0.22	0.12
32 μM K + 32 μM DPU	0.12	1.83	1.36

*Average of eight flasks.

**In previous passage, tissues did not grow.

cytokinin-autonomous growth. Tissues from these two genotypes that had been grown on optimal or close to optimal kinetin concentrations remained cytokinin dependent. Culture from these two genotypes that had been exposed to DPU in the previous passage exhibited vigorous cytokinin-autonomous growth and higher concentrations of kinetin were required to suppress the autonomous growth habit than in the case of callus tissues grown on kinetin alone. Callus tissue derived from genotype P.I. 260415 remained cytokinin dependent regardless of the composition of the medium in the previous passage.

6. The cytokinin activities of ureidopurines.

N^6-ureidopurines, purine derivatives in which an N^6-side chain is linked to the 6-position of the purine ring by a -NH-CO-NH- bridge, combine structural features of both the cytokinin-active urea derivatives and the purine cytokinins. Therefore, it was of interest to examine the effect of these compounds on P. lunatus callus cultures. The cytokinin activities of three ureidopurines (N^6-phenylureidopurine, N^6-p-tolylureidopurine, and N^6-cyclohexylureidopurine) have been determined and compared with the activities of N^6-benzylaminopurine, N^6-cyclohexylmethylaminopurine, and DPU in callus cultures of P. lunatus cv. Kingston and cv. Jackson Wonder. As shown in Figures 9 and 10, in both genotypes N^6-benzylaminopurine and N^6-cyclohexylmethylaminopurine were more active than any of the ureidopurines tested. The three ureidopurines exhibited the same relative activities in both callus tissue lines (N^6-phenylureidopurine > N^6-p-tolylureidopurine > N^6-cyclohexyl-

Figure 9. A comparison of the cytokinin activities of 6-ureidopurines, benzylaminopurine, and 6-cyclohexylmethylaminopurine in promoting callus growth of P. lunatus cv. Kingston.

Stock tissues, in first passage were grown on the medium containing 2.5 μM picloram and 5 μM kinetin for 21 days. Cytokinins were dissolved in dimethyl sulfoxide and added to the autoclaved medium (0.025 ml/flask). The tissues were harvested and weighed after 35 days. The average fresh callus weight of the four replicate flasks was determined. Abbreviations on the Figure were N^6-benzyl-aminopurine (BAP), N^6-cyclohexylmethylaminopurine (CMP), N^6-phenylureidopurine (PUP), N^6-p-tolylureidopurine (TUP), N^6-cyclohexylureidopurine (CUP), and N,N'-diphenylurea (DPU).

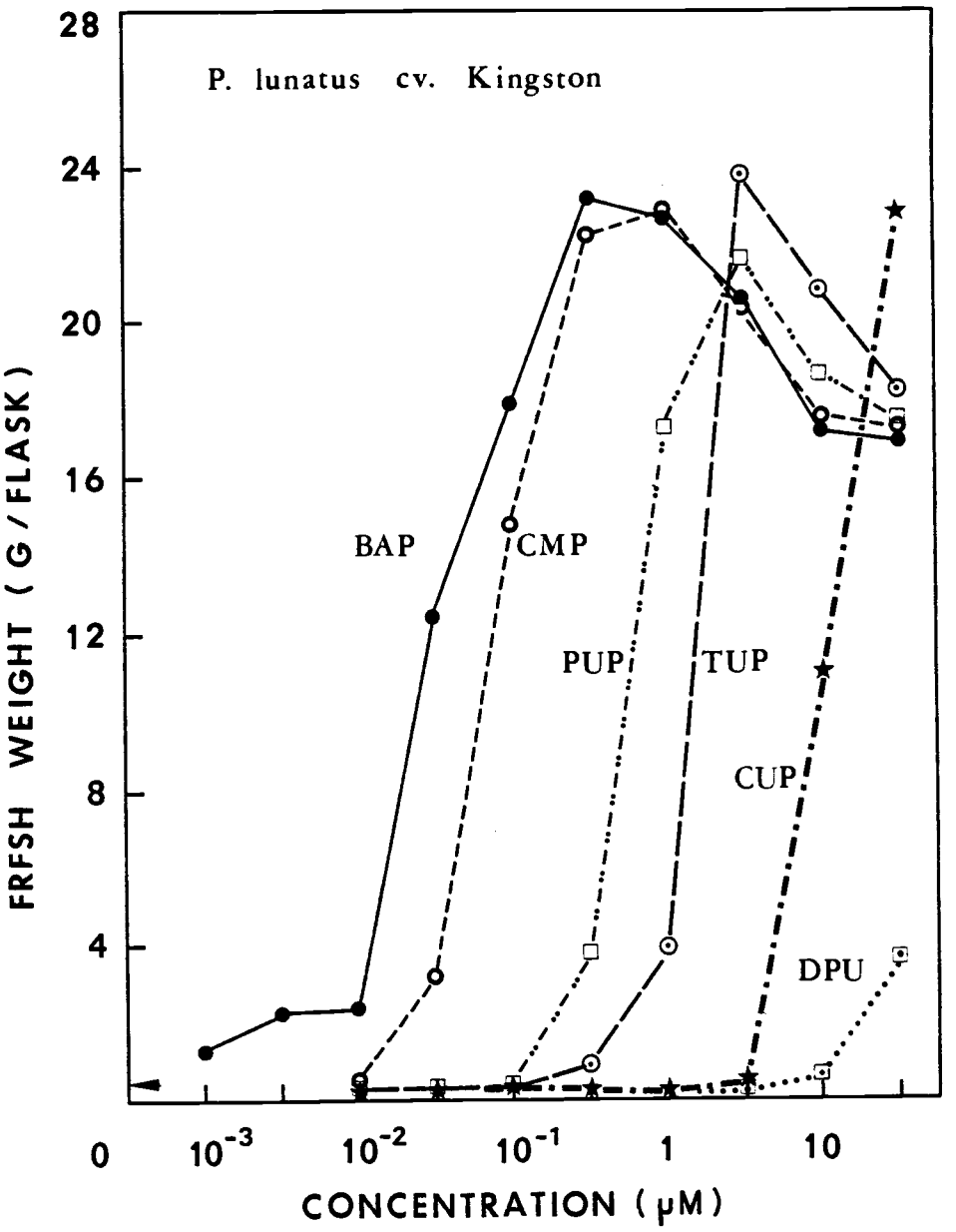
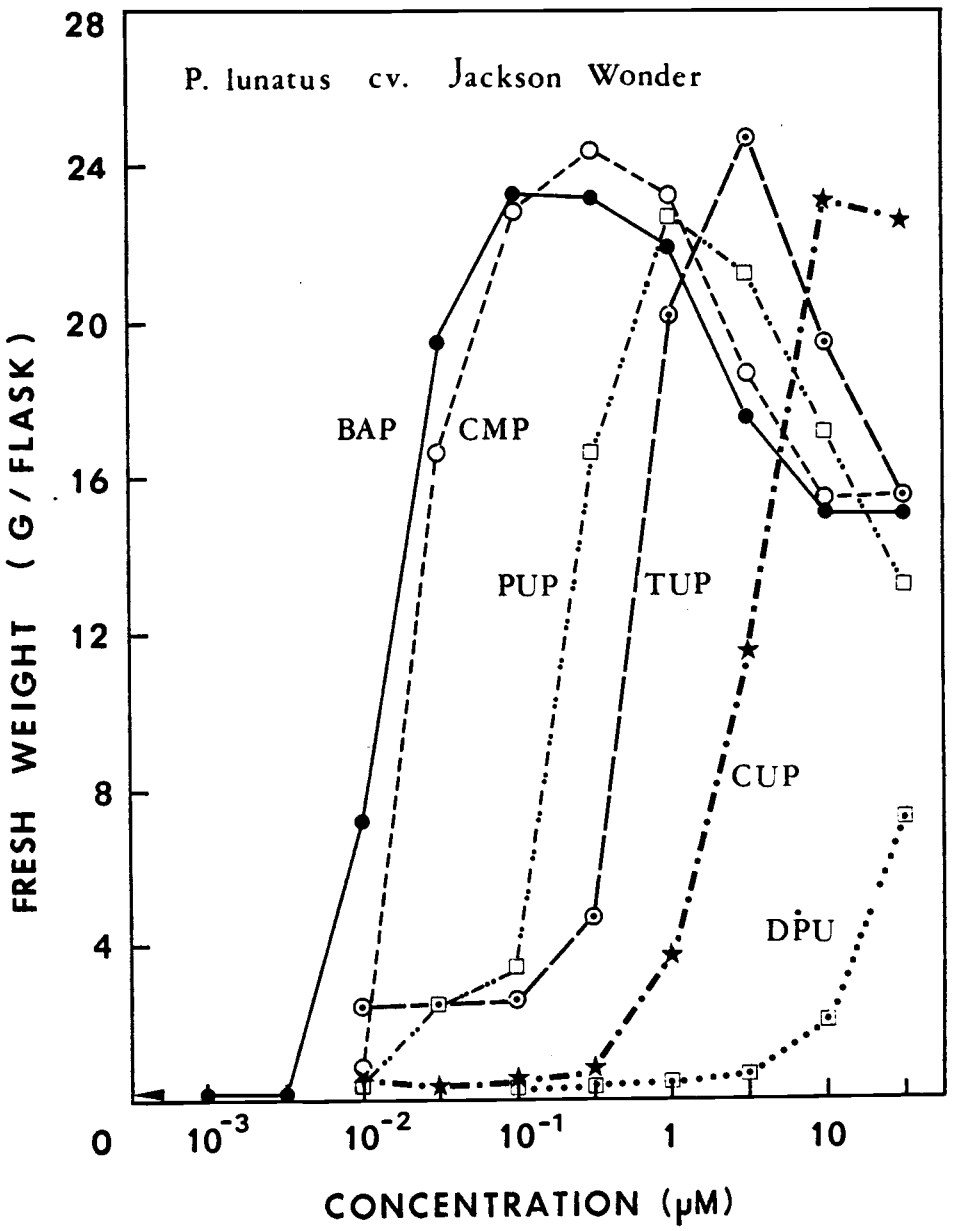

P. lunatus cv. Kingston
FRFSH WEIGHT (G / FLASK)
28
24
20
16
12
8
4
0
BAP
CMP
PUP
TUP
CUP
DPU
10^{-3}
10^{-2}
10^{-1}
1
10
CONCENTRATION (μM)

Figure 10. A comparison of the cytokinin activities of 6-ureidopurine, benzylaminopurine, and 6-cyclohexylmethylaminopurine in promoting callus growth of P. lunatus cv. Jackson Wonder.

Stock tissues, in first passage, were grown on the medium containing 2.5 μM picloram and 5 μM kinetin for 21 days. Cytokinins were dissolved in dimethyl sulfoxide and added to the autoclaved medium (0.025 ml/flask). The tissues were harvested and weighed after 35 days. The average fresh weight of the four replicate flasks was determined. Abbreviations on the Figure were N^6-benzylaminopurine(BAP), N^6-cyclohexylmethylaminopurine (CMP), N^6-phenylureidopurine (PUP), N^6-p-tolylureidopurine (TUP), N^6-cyclohexylureidopurine (CUP), and N,N'-diphenylurea (DPU).


P. lunatus cv. Jackson Wonder
FRESH WEIGHT (G/FLASK)
CONCENTRATION (μM)
BAP
CMP
PUP
TUP
CUP
DPU
28
24
20
16
12
8
4
0
10^-3
10^-2
10^-1
1
10

ureidopurine), at appropriate concentrations, all ureidopurines tested promoted vigorous growth and maximal yields of callus tissue. Callus growth on DPU-containing media was rather irregular and low yields were obtained at a concentration of 32μM DPU.

To test the ability of these callus tissues to exhibit cytokinin-autonomous growth, callus tissues from 25-day-old cultures were transferred to cytokinin-free media. The results shown in Table 7 indicate that callus tissues previously grown on suboptimal and supraoptimal concentrations of N^6-cyclohexylureidopurine, N^6-phenylureidopurine, and N^6-p-tolylureidopurine as well as benzylaminopurine and N^6-cyclohexylmethylaminopurine could proliferate in the absence of cytokinin in the media. At optimal or close to optimal concentrations, all of these cytokinin-active compounds suppressed the development of cytokinin-autonomous growth. Thus, the behavior of P. lunatus callus tissues in response to N^6-ureidopurines appears similar to the response of the tissues to other N^6-substituted purine derivatives.

Table 7. Effect of ureidopurines in inducing cytokinin-autonomy in callus tissues derived from P. lunatus genotypes cv. Kingston and cv. Jackson Wonder.

Treatment in second passage (μM)	Kingston		Jackson Wonder	
	Fresh weight (g/flask)*			
	Second-passage	Cytokinin-free medium	Second-passage	Cytokinin-free medium
N,N'-diphenylurea				
10	0.54	5.36	1.98	11.74
32	3.60	5.75	7.24	13.51
Benzylaminopurine				
0.01			7.12	10.69
0.03	12.60	0.23	19.61	6.80
0.1	17.80	0.20	23.23	0.44
0.3	23.13	0.13	23.24	0.08
1	22.69	0.09	22.01	0.08
3	20.52	0.12	17.49	1.33
10	17.29	0.95	15.07	3.27
32	16.95	3.41	15.14	5.29
N^6-cyclohexylmethylaminopurine				
0.03	3.17	1.23	16.63	9.00
0.1	15.65	0.09	22.83	0.09
0.3	22.23	0.09	24.33	0.08
1	22.87	0.09	23.33	0.08
3	20.28	0.33	18.66	0.09
10	17.39	1.13	15.29	5.81
32	17.34	2.08	15.67	9.52
N^6-phenylureidopurine				
0.3	3.78	3.56	16.69	1.43
1	17.32	2.78	22.73	0.09
3	21.73	0.11	21.11	3.80
10	18.59	2.71	17.15	7.25
32	17.56	5.10	13.34	9.42
N^6-cyclohexylureidopurine				
3			11.39	12.11
10	11.01	0.09	22.99	0.51
32	22.92	0.09	22.60	0.93
N^6-p-tolylureidopurine				
0.1			2.48	12.19
0.3			4.70	3.19
1	3.87	1.35	20.05	1.03
3	23.83	0.10	24.73	0.28
10	20.87	0.39	19.45	6.48
32	18.29	3.19	15.41	7.96

*Average fresh weights (g/flask) of four flasks were determined.

B. Influence of N,N'-diphenylurea on the growth of callus tissue from Nicotiana tabacum L. cv. Wisconsin 38.

For comparison with the results obtained with P. lunatus callus cultures, the effect of DPU on the growth of tobacco callus tissue was examined. The fresh weights of callus tissues of Nicotiana tabacum cv. Wisconsin 38 after a growth period of five weeks on DPU-containing media are shown in Figure 11. The tobacco callus tissue grew uniformly in response to DPU and showed optimal growth at DPU concentrations of 10 μM and 32 μM. The activity of DPU in promoting the growth of tobacco tissue was approximately 100 fold less than that of kinetin. The uniform response of tobacco callus tissue to DPU contrasted with the irregular and much weaker responses of callus tissues of P. lunatus genotypes.

To determine whether tobacco tissue grown on media containing DPU had gained the capacity for cytokinin-autonomous growth, 25-day-old callus tissue was transferred from DPU containing media to cytokinin-free media. As shown in Figure 12, only a slight and irregular increase in background growth was observed on the cytokinin-free media, even after several transfers of the tobacco callus tissue on DPU-containing media.

The P. lunatus callus cultures used in the work reported here are grown on picloram as an auxin source, while tobacco callus tissues are usually grown on media containing indole-3-acetic acid. The possibility that the difference in auxin source might account for differences in the response of the two tissues to cytokinin was,

Figure 11. Effect of N,N'-diphenylurea in promoting callus growth of N. tabacum cv. Wisconsin 38.

Stock callus tissues were previously grown on 0.15 μM kinetin-containing medium for 24 days. After a culture period of five weeks on a range of DPU-containing medium, The callus tissues were harvested and the average fresh weights (g/flask) of eight flasks are given at the right of the figure.

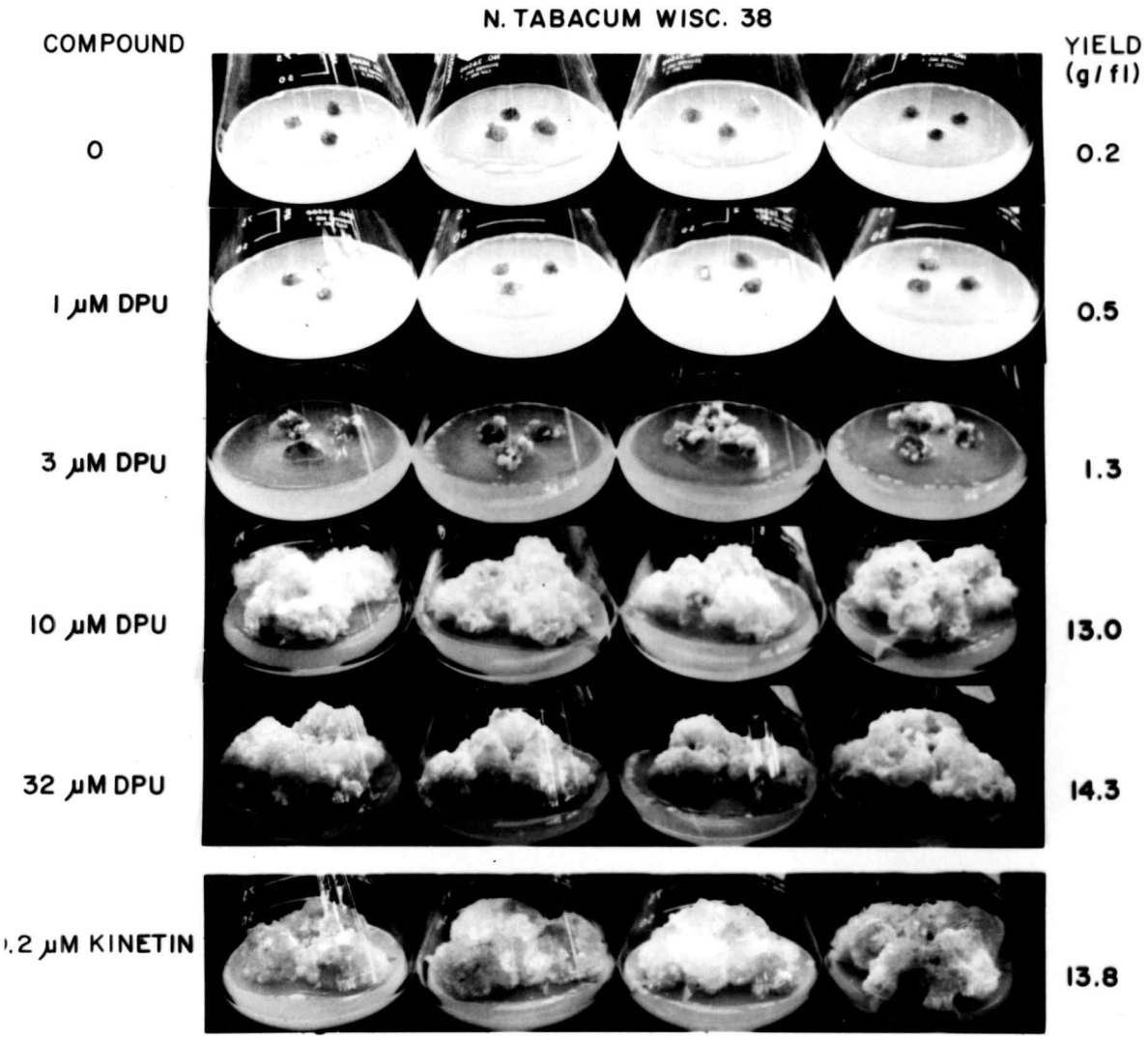
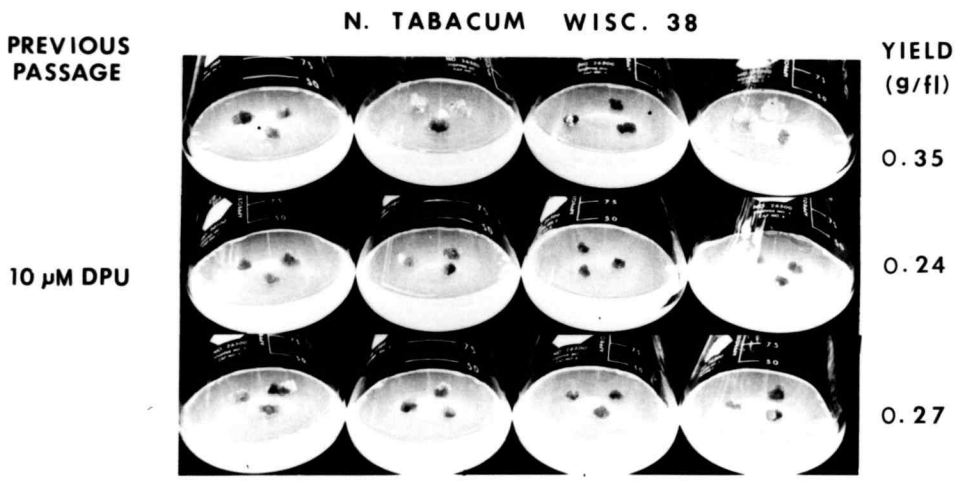

N. TABACUM WISC. 38
COMPOUND
YIELD (g/fl)
0
0.2
1 µM DPU
0.5
3 µM DPU
1.3
10 µM DPU
13.0
32 µM DPU
14.3
.2 µM KINETIN
13.8

Figure 12. Effect of N,N-diphenylurea in inducing cytokinin autonomy of N. tabacum cv. Wisconsin 38.

Tobacco callus tissues grown on 10 μM DPU-containing medium were tested for their habituation on cytokinin-free medium. The callus tissues were harvested after 35 days culture period and the average fresh weights (g/flask) of four flasks are given at the right of the figure.


N. TABACUM WISC. 38
PREVIOUS PASSAGE
10 μM DPU
YIELD (g/fl)
0.35
0.24
0.27

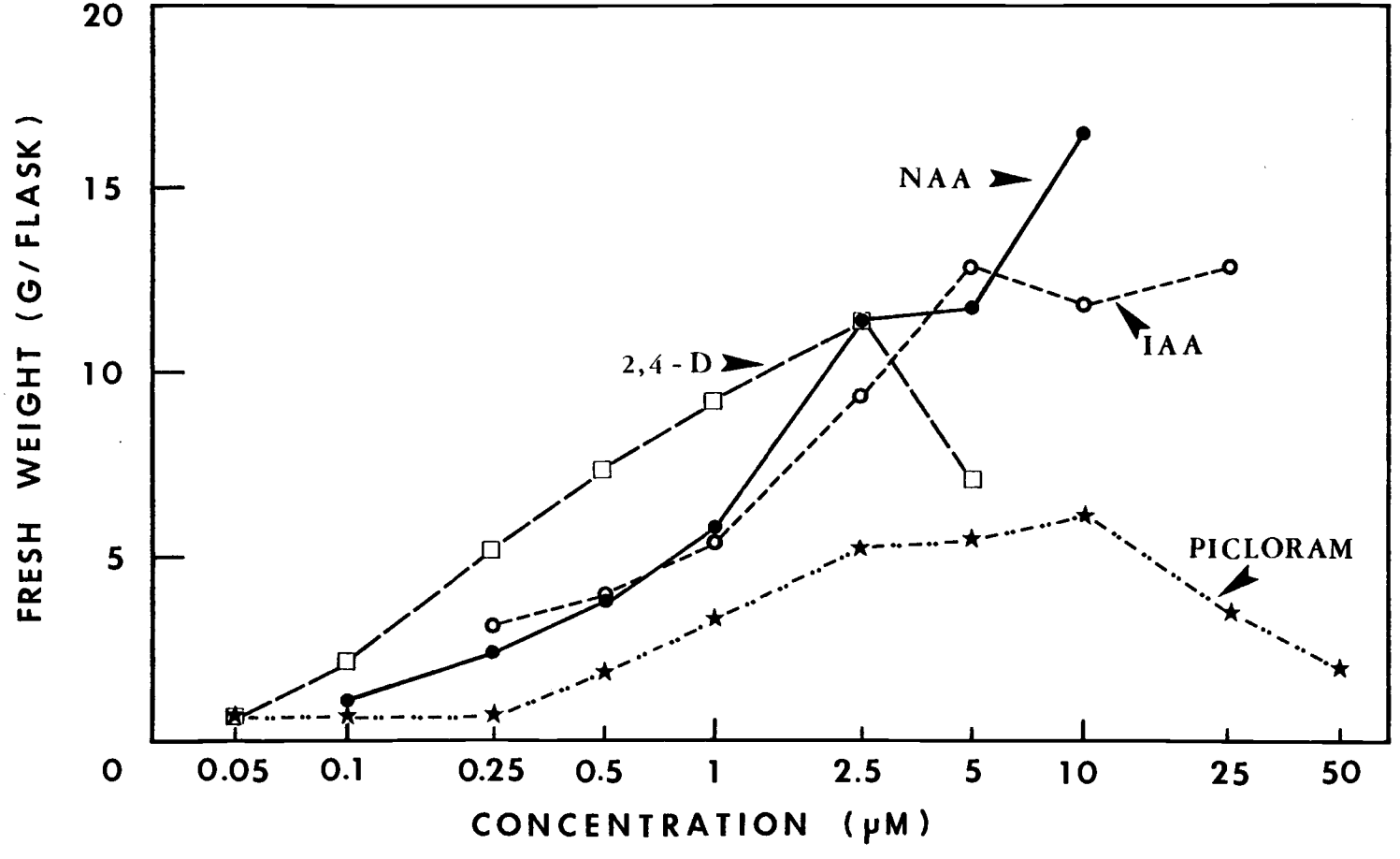
therefore, examined. In initial experiments, the response of tobacco callus tissue to four auxins (indole-3-acetic acid, 2.4-dichlorophenoxy-acetic acid, naphthalene acetic acid, and picloram) was tested using kinetin (0.15 μM) as a cytokinin. The appropriate amounts of each auxin were dissolved in dimethyl sulfoxide and added to each flask (0.05 ml auxin solution per 50 ml medium) after autoclaving. The tissues were harvested and weighed after 35 days. The results are shown in Figure 13.

Tobacco callus tissue exhibited a relatively weak response to picloram, the auxin used in Phaseolus tissue cultures (Mok and Mok, 1977). Maximum growth of tobacco callus was obtained at relatively high concentration of picloram (10 μM), and the total yield of callus tissue was low at this concentration as compared with the other auxins. Optimal levels of 2,4-dichlorophenoxyacetic acid for growth of tobacco tissue were between 1 μM and 2.5 μM. The growth of tissue was inhibited at concentrations higher than 2.5 μM. Indole-3-acetic acid and naphthalene acetic acid both stimulated callus growth over a wide range of concentrations. The most rapid callus growth occurred at 10 μM NAA and 25 μM IAA, respectively. The highest yield of callus were obtained on medium supplemented with 10 μM of NAA.

Tobacco callus tissues grown for 25 days on the two best concentrations of each of the four different auxins: [IAA (10 μM, 25 μM), NAA (5 μM, 10 μM), 2,4-D (1 μM, 2.5 μM), and picloram (5 μM, 10 μM)] were transferred to the medium containing the same auxin concentrations and either 10 μM DPU or 0.15 μM kinetin. After 25 days growth, the tobacco tissues were then transferred to cytokinin-free media.

Figure 13. Effect of different auxins on callus growth of Nicotiana tabacum cv. Wisconsin 38.

Tobacco callus tissue previously grown on RM-1965 medium containing indole-3-acetic acid (2 mgl/l) and 0.14 μM kinetin for 24 days were used as stock tissue. Response of the tissue to four different auxins, picloram, 2,4-dichlorophenoxyacetic acid, naphthalene acetic acid, and indole-3-acetic acid was tested. Auxins were dissolved in dimethyl sulfoxide and added to the autoclaved medium (0.05 ml/flask). Three pieces of callus tissue, about 25 mg each, were planted. The tissues were harvested and weighed after 35 days. The average fresh weight of the four replicate flasks was determined.


FRESH WEIGHT (G/FLASK)
20
15
10
5
0
0.05
0.1
0.25
0.5
1
2.5
5
10
25
50
CONCENTRATION (μM)
NAA
2,4 - D
IAA
PICLORAM

As shown in Table 8, there was no particularly strong influence of the type of auxin used on the ability of the callus tissue to grow on cytokinin-free media. Although some of the callus tissues exhibited increased growth on the cytokinin-free media, in no case did the yield of callus tissue approach the yield observed with P. lunatus callus tissues after exposure to DPU.

C. Development of methodology for detection of in vivo cytokinin biosynthesis in callus tissues.

1. Initial attempts to detect cytokinin biosynthesis in tobacco callus tissue.

To determine the nature of the effect of DPU and purine cytokinins on endogenous cytokinin biosynthesis in callus tissues of P. lunatus and N. tabacum, it is desirable to have a more direct measure of cytokinin biosynthesis than the growth response of the tissues. Preliminary work on the detection of cytokinin biosynthesis from adenine-8-^{14}C was undertaken with the cytokinin-autonomous strain of tobacco callus tissue used by Einset and Skoog (1973).

The callus cultures were grown for 25 days prior to use in labelling experiments, and the yield of tissue was approximately 10 grams per flask containing 50 ml of medium. The label (2 μCi of adenine-8-^{14}C) was supplied to the tissue as a 1 ml aqueous solution which was distributed in droplets over the surface of the callus tissue and was quickly absorbed by the tissue. After varying periods of incubation, the tissue was homogenized in two and one-half volumes of

Table 8. Effect of four different auxins (IAA, NAA, 2,4-D, and Picloram) in inducing cytokinin autonomy in callus cultures of Nicotiana tabacum L., var. Wisconsin #38.

Auxin	Fresh weight (g/flask)*	
	0.15 μM kinetin in previous passage	10 μM DPU in previous passage
10 μM IAA**	0.43	0.74
25 μM IAA	1.16	2.07
5 μM NAA	1.15	1.51
10 μM NAA	0.68	1.95
1.0 μM 2,4-D	0.45	0.32
2.5 μM 2,4-D	1.06	1.57
5 μM Picloram	0.66	0.25
10 μM Picloram	1.32	0.58

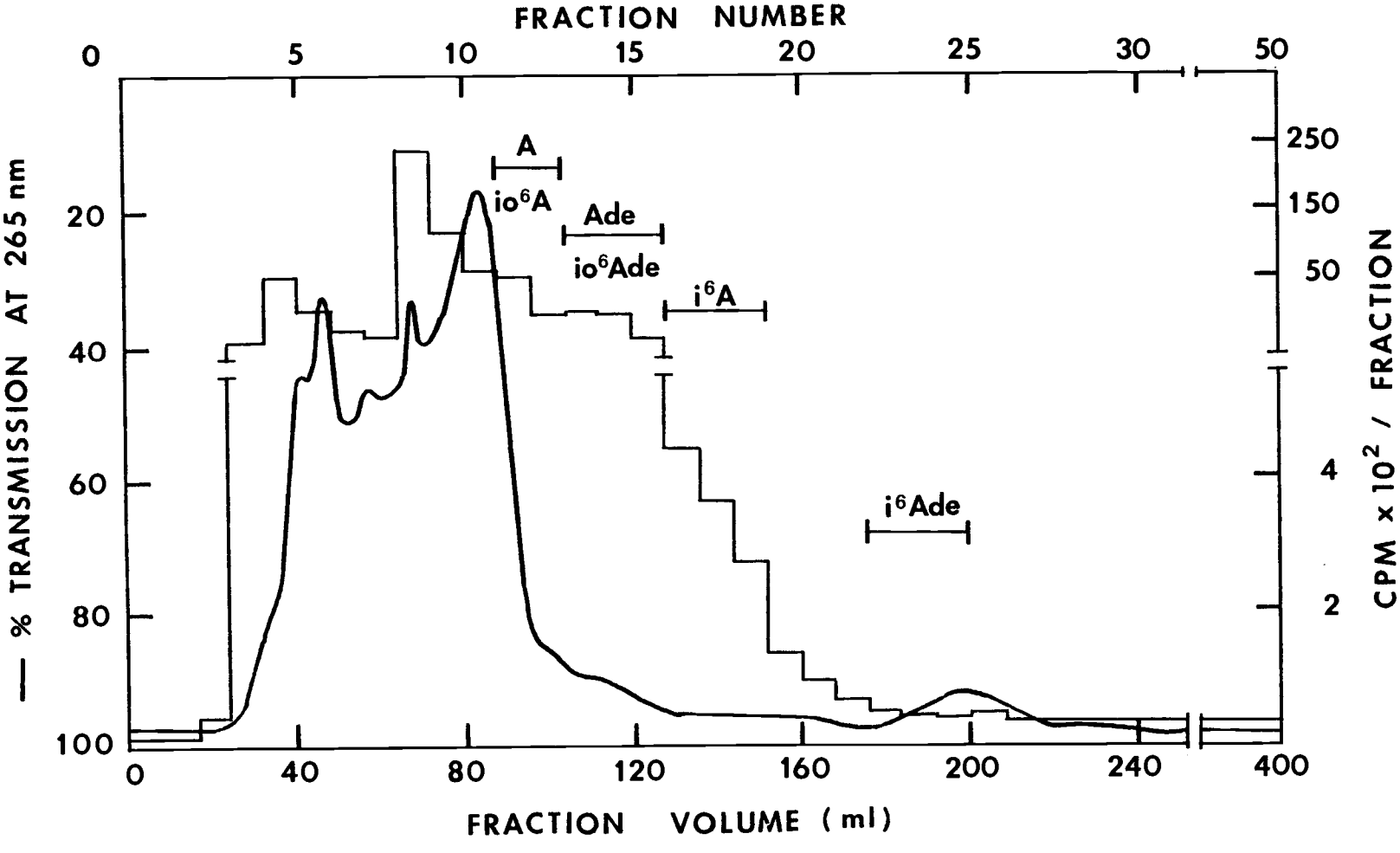
*Average of eight flasks.

**In habituation test, indicated concentrations of auxins were included on the cytokinin-free-medium.

ethanol, the debris removed by filtration, and the ethanol extract taken to dryness *in vacuo* at 37°C. The solids recovered in this manner were redissolved in a small volume of 33% (v/v) ethanol, the solution clarified by centrifugation, and the supernatant chromatographed on a Sephadex LH-20 column in the same solvent. The elution profile of a representative Sephadex LH-20 fractionations after a 5 hours labeling period is shown in Figure 14. The label was distributed throughout the first two bed volumes of the elution profile and no discrete peaks of label could be detected, although some improvement in resolution could be obtained by rechromatographing pooled fractions from this column on Sephadex LH-20 in distilled water. Under these conditions zeatin is resolved from adenine and ribosylzeatin from adenosine. However, the large amount of label associated with adenine and adenosine made it impossible to be certain that discrete peaks of label were present in fractions corresponding to the elution positions of cytokinin standards. On the basis of this result, it was decided to pursue methods for preliminary purification of ethanol extracts of the callus tissues to try to eliminate some of the background of label that was not associated with cytokinin-active compounds. Although Dowex 50 and other cation exchange columns have been used to partially purify cytokinin from crude plant extracts, the use of such columns necessitates exposure of the extracts to rather harsh conditions and introduces problems in achieving complete recovery (Vreman and Corse, 1975). Therefore, it was decided to explore the use of two hydrophobic, non-ionic resins Amberlite XAD-2 and Amberlite XAD-7. These materials are

Figure 14. Distribution of radioactivity in a crude ethanol extract of cytokinin-autonomous tobacco callus tissue incubated with adenine-8-^{14}C for 5 hours.

An ethanol extract was prepared from the tobacco callus tissue after incubation with 2 μCi of adenine-8-^{14}C for 5 hours. Solids recovered from the ethanol extract were dissolved in 2 ml of 33% (v/v) ethanol and fractionated on a Sephadex LH-20 column (20 g, 1.5 x 50 cm) in the same solvent. Eight-ml fractions were collected at a flow rate of about 24 ml per hour. Cytokinin standards were later chromatographed on the same column. Half of each fraction was counted for radioactivity measurements.


FRACTION NUMBER
0
5
10
15
20
25
30
50
A
io⁶A
Ade
io⁶Ade
i⁶A
i⁶Ade
— % TRANSMISSION AT 265 nm
0
20
40
60
80
100
CPM x 10² / FRACTION
250
150
50
4
2
0
40
80
120
160
200
240
400
FRACTION VOLUME (ml)

readily available and rather inexpensive, characteristics which make them potentially suitable for large scale as well as small scale applications.

2. Recovery of cytokinin standards from Amberlite XAD columns.

Amberlite XAD-2 and XAD-7 were initially tested for their effectiveness in binding cytokinins as well as other purines and pyrimidines. The compounds were applied to the columns in aqueous solutions, the columns washed with additional water, and any bound compounds were then eluted with 70% (v/v) ethanol. The results of model experiments using various cytokinin standards, bases, and ribonucleosides are shown in Table 9 and Table 10. All the cytokinins tested were bound to the resins, and were not removed by extensively washing with water. Other bases and nucleosides were more weakly bound and were eluted from the column to varying degrees by the water wash. Futhermore, the cytokinins could be recovered essentially quantitatively by elution with 70% (v/v) ethanol. Most cytokinins were removed from the column within five bed volumes and even completely recovered within 10 bed volumes. Amberlite XAD-7 bound the free base form of the cytokinins rather strongly as well as most of the adenine applied to the column. Therefore, further testing was restricted to XAD-2.

Table 9. Percentage recovery of nucleosides and cytokinins from Amberlite XAD-2 column with 70% (v/v) ethanol.

Compound	Sample-* flow-through	Water-** washing	1-5 bed volumes (70% ethanol)	6-10 bed volumes (70% ethanol)	Total recovery(%)
Adenine	80	18	3	0	101
Adenosine	37	16	44	3	100
Guanosine	62	23	14	1	100
Uridine	82	14	3	1	100
Cytidine	90	10	2	1	103
io^6Ade	1	1	100	1	103
io^6A	1	1	98	2	102
i^6Ade	0	0	92	8	100
i^6A	0	0	97	7	104
BAP	1	0	88	8	97
BAPR	1	1	93	7	102

*100 ml of an 0.02 mM aqueous solution was applied to an Amberlite XAD-2 column (17 g, 0.9 x 30 cm), separately.

**Ten bed volumes of water were collected, evaporated to dryness, and redissolved in appropriate amounts of distilled water for the purpose of measuring recovery.

Table 10. Percentage recovery of nucleosides and cytokinins from Amberlite XAD-7 column with 70% ethanol.

Compound	Sample-* flow-through	Water-** washing	1-5 bed volumes (70% ethanol)	6-10 bed volumes (70% ethanol)	Total recovery(%)
Adenine	2	6	92	2	103
Adenosine	7	16	75	3	101
Guanosine	48	34	15	3	100
Uridine	69	21	9	2	101
Cytosine	66	26	8	4	104
io^6Ade	2	1	82	15	100
io^6A	2	1	92	7	102
i^6Ade	1	0	94	5	100
i^6A	1	1	92	6	100
BAP	0	0	43	43	86
BAPR	1	1	90	9	101

*100 ml of an 0.02 mM aqueous solution was applied to an Amberlite XAD-7 column (17 g, 0.9 x 30 cm), separately.

**Ten bed volumes of water were collected and evaporated to dryness and redissolved in appropriate amounts of distilled water for the purpose of measuring recovery.

3. Recovery of cytokinins from tRNA hydrolysates using Amberlite XAD-2 columns.

To provide a more realistic test of the utility of Amberlite XAD-2 in the initial purification of naturally occurring cytokinins, the recovery of cytokinin ribonucleosides from hydrolysates of wheat germ, Escherichia coli, and yeast tRNA samples was examined. The tRNA samples were enzymatically hydrolyzed to nucleosides. The hydrolysates were adjusted to pH 5.8 and applied to Amberlite XAD-2 columns. The columns were washed extensively with distilled water and eluted with 70% (v/v) ethanol. Samples of the column eluates were acid-hydrolyzed as described in Materials and Methods and tested for cytokinin activity in the tobacco bioassay. The results are shown in Table 11. The data show that the recovery of cytokinin activity was essentially quantitative. As E. coli tRNA is known to contain 2-methylthio-N^6-(Δ^2-isopentenyl)adenosine (Nishimura et al., 1969), it appears that the method is also suitable for recovery of the methylthiolated cytokinins. Recovery was complete in all cases within 10 bed volumes of 70% (v/v) ethanol.

4. Recovery of cytokinins from Agrobacterium tumefaciens C58 culture filtrate by using Amberlite XAD-2.

To further test the utility of this procedure for use in cytokinin purification, the recovery of cytokinin activity from 5 liters of culture filtrate from Agrobacterium tumefaciens strain C58 was examined. The results are shown in Table 12. The crude culture filtrate and

Table 11. Recovery of cytokinin activity from tRNA hydrolysates by trace enrichment on Amberlite XAD-2.

Sample	Cytokinin activity(μg KE/100 $_{260}$ units tRNA)		
	E. coli	Brewer's yeast	Wheat germ
tRNA hydrolysate (crude)	5.08	5.15	0.57
Sample column-through	—*	—	—
Water-washing (10 bed volumes)	—	—	—
1-5 bed volumes (70% ethanol)	5.10	5.39	0.63
6-10 bed volumes (70% ethanol)	0.27	0.02	0.01
Other 5 bed volumes (95% ethanol)	—	—	—

*Bars indicate no cytokinin activity in tobacco tissue bioassay.

Cytokinin activity is expressed as Microgram kinetin Equivalents (μg KE) as defined in Materials and Methods.

the material that washed through the column during loading was toxic when tested directly in the tobacco bioassay. Therefore, these materials were evaporated to dryness and cytokinin activity recovered by extracting the solids with appropriate amounts of water-saturated ethyl acetate: t-butanol (2:1, v/v). The 70% (v/v) ethanol eluates from the Amberlite XAD-2 column did not require solvent extraction prior to bioassay and were tested directly. Most of the cytokinin activity bound to the column and eluted in the first five bed volumes of 70% (v/v) ethanol. The material recovered from the XAD-2 column by elution with 70% (v/v) ethanol was chromatographed on a Sephadex LH-20 column in 33% (v/v) ethanol (Figure 15). Fractions were pooled according to the elution volumes of cytokinin standards and 67% aliquots were bioassayed for cytokinin activity. Cytokinin activity was distributed throughout the elution profile, but most of the activity was found in two fractions, one corresponding to i^6Ade (VII, 2.95 KE/ 1,000 ml culture filtrate), and the other to io^6Ade (IV, 2.35 KE/ 1,000 ml culture filtrate). The cytokinin activity of these two fractions accounted for more than 88% of total activity in the A. tumefaciens culture filtrate.

5. Recovery of cytokinins from cytokinin-autonomous callus tissue of Phaseolus lunatus cv. Kingston using Amberlite XAD-2.

Extracts of plant tissues pose much more difficult problem in cytokinin purification than either tRNA hydrolysates or bacterial culture filtrates. To test the utility of Amberlite XAD-2 with this

Table 12. Recovery of cytokinin activity from culture filtrates of Agrobacterium tumefaciens C58 by trace enrichment on Amberlite XAD-2.

Sample	Cytokinin activity (µg KE per 1,000 ml filtrate)
Culture filtrate(crude)*	5.67
XAD-2 column sample* flow through	0.34
Water-washing* (10 bed volumes)	—**
1-5 bed volumes (70% ethanol)	5.83
6-10 bed volumes (70% ethanol)	0.05
Total cytokinin activity of Sephadex LH-20 fraction	6.01

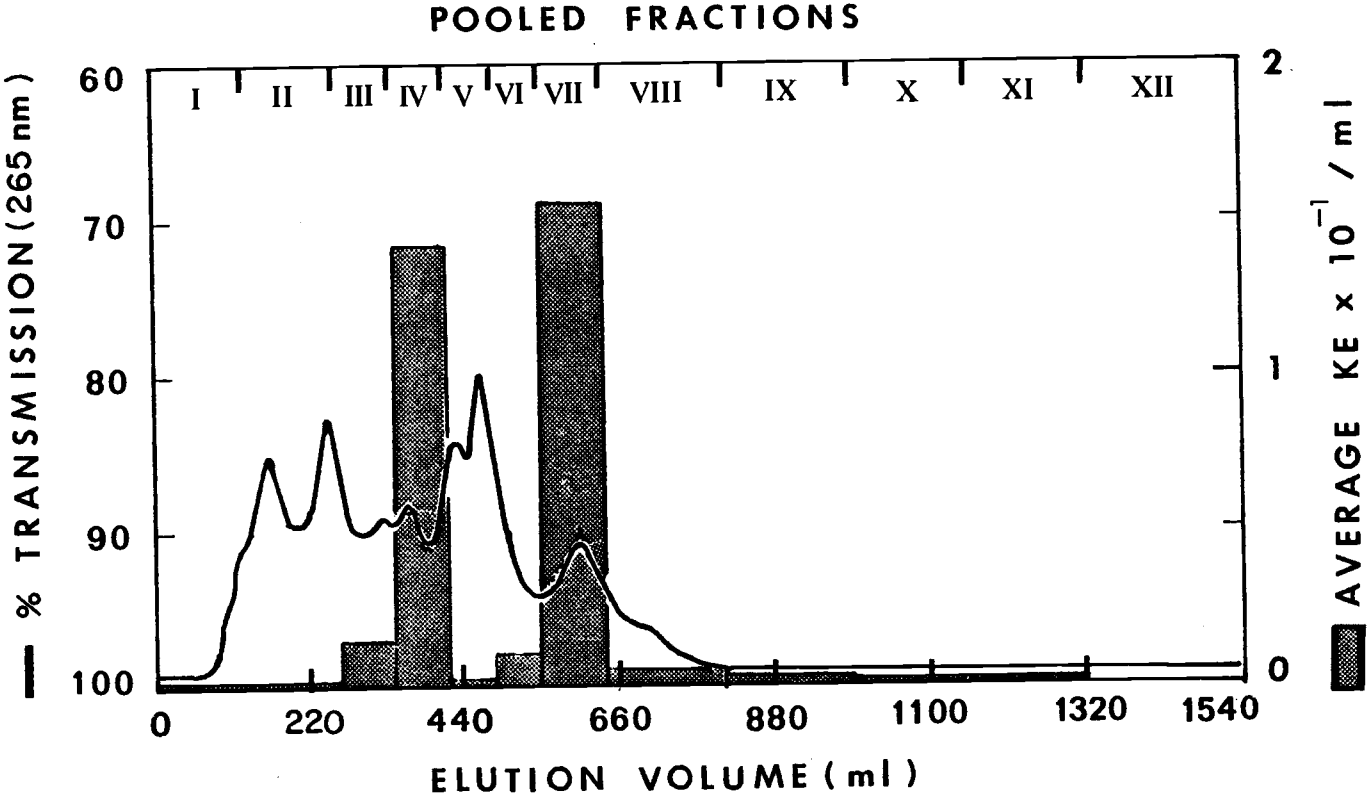
*After sample was evaporated in reduced pressure and extracted six times with 20 ml of water-saturated ethyl acetate: t-butanol (2:1, v/v).

**Bars indicate no cytokinin activity in tobacco bioassay.

Cytokinin activity is expressed as Microgram Kinetin Equivalents (µg KE) as defined in Materials and Methods.

Figure 15. Distribution of cytokinin activity in culture filtrate of Agrobacterium tumefaciens C58.

Culture filtrate (4,700 ml) of Agrobacterium tumefaciens C58 was initially purified on an Amberlite XAD-2 column (200 g, 2.5 x 50 cm)as described in Materials and Methods. The solids recovered from the 70% ethanol eluate were dissolved in 4.7 ml of 33% (v/v) ethanol, applied to a Sephadex LH-20 column (60 g, 2.5 x 45 cm), and eluted with the same solvent. Eleven-ml fractions were collected at a flow rate of 35 ml per hour. Cytokinin standards were later run on the same column, and the fractions were pooled as shown. Aliquots (67% of each pooled fraction) were tested for cytokinin activity in the tobacco callus bioassay.


POOLED FRACTIONS
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
60
70
80
90
100
% TRANSMISSION (265 nm)
2
1
0
AVERAGE KE x 10⁻¹ / ml
0
220
440
660
880
1100
1320
1540
ELUTION VOLUME (ml)

type of material, an attempt was made to examine the cytokinin constituents of a cytokinin-autonomous strain of callus tissue derived from P. lunatus cv. Kingston. Approximately 4,000 g of autonomous callus tissue (about 10 g/flask) of P. lunatus cv. Kingston, grown for 26 days on cytokinin-free medium containing 2.5 μM picloram as an auxin source was homogenized in 2½ volumes of 95% (v/v) ethanol. The ethanol was removed by evaporation at 37°C in vacuo and the remaining aqueous solution applied to an Amberlite XAD-2 column. After extensive washing with water, the column was eluted with 70% (v/v) ethanol (10 bed volumes) and the ethanol eluate evaporated to dryness. The solids recovered in this fashion were highly colored. The solids were then extracted six times with water-saturated ethyl acetate followed by six extractions with ethyl acetate: t-butanol (2:1, v/v). The two extracts were chromatographed on separate Sephadex LH-20 columns in 33% ethanol. The results are shown in Figure 16 and Figure 17, respectively. Cytokinin activity with elution volumes corresponding to the glucose esters of zeatin and ribosylzeatin (I, II), ribosylzeatin (III), and zeatin (IV) was detected. Pooled fractions, VI, VIII, X, and XI also had significant cytokinin activity. Among the known naturally occurring cytokinins only the methylthiolated cytokinins and the side chain glycosylated derivatives of zeatin exhibit such late elution on Sephadex LH-20. No evidence was obtained for the presence of either N^6-isopentenyladenosine (V) or N^6-isopentenyladenine (VII) activity recovered from the tissue. The total amount of cytokinin corresponded to approximately 0.4 μg KE per kg fresh weight (Table 13).

Figure 16. Distribution of cytokinin activity in an ethanol extract prepared from a cytokinin-autonomous callus tissue line of P. lunatus cv. Kingston: Fractionation of material recovered after water-saturated ethyl acetate extraction.

Cytokinin-autonomous tissue of P. lunatus cv. Kingston (4 kg) was extracted with 2½ volume of cold 95% ethanol. Ethanol extract was passed through an Amberlite XAD-2 column (140 g, 2.5 x 30 cm) previously equilibrated with 70% (v/v) ethanol and the ethanol then removed by evaporation. The aqueous solution thus obtained was diluted to the original volume of the ethanol extract and applied to an Amberlite XAD-2 column (140 g, 2.5 x 30 cm). The column was washed with 30 bed volumes of water and eluted with 10 bed volumes of 70% (v/v) ethanol. The dry solids recovered from the ethanol eluate were extracted with water-saturated ethyl acetate as described in Materials and Methods. Extract was taken to dryness and the solids dissolved in 5 ml of 33% (v/v) ethanol and fractionated on a Sephadex LH-20 column (40 g, 2 x 57 cm) in the same solvent. Further procedures were described in legend of Fig. 17.

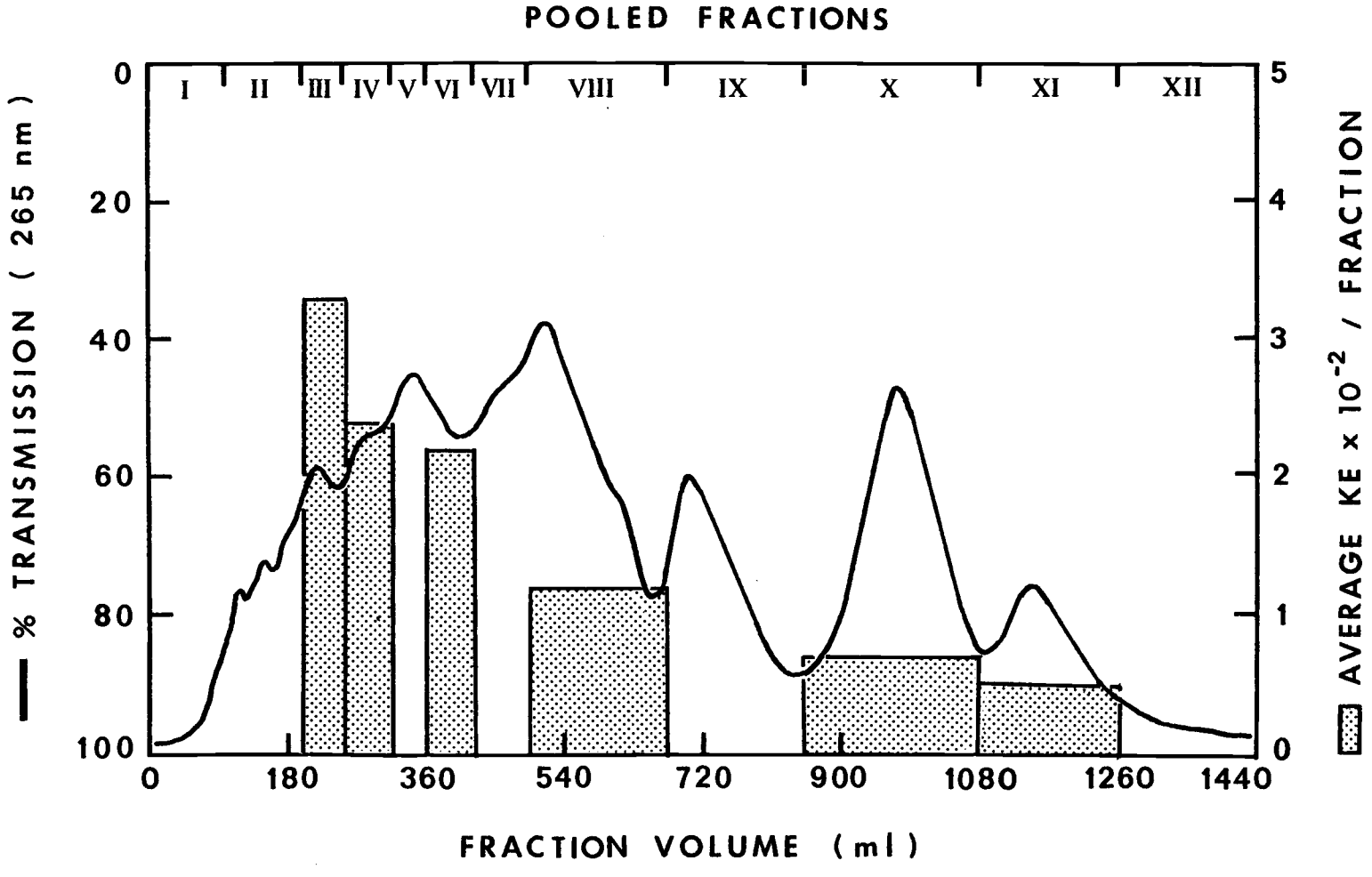

POOLED FRACTIONS
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
0
20
40
60
80
100
% TRANSMISSION (265 nm)
5
4
3
2
1
0
AVERAGE KE x 10-2 / FRACTION
0
180
360
540
720
900
1080
1260
1440
FRACTION VOLUME (ml)

Figure 17. Distribution of cytokinin activity in an ethanol extract prepared from a cytokinin-autonomous callus tissue line of P. lunatus cv. Kingston: Fractionation of material recovered after ethyl acetate : t-butanol extraction.

An ethanol extract of the callus tissue was prepared and partially purified by Amberlite XAD-2 treatment and solvent extraction as described in Materials and Methods and legand to Figure 16. The residue of H_2O-saturated ethyl acetate extraction was dried, and extracted six times with 20 ml of ethyl acetate : t-butanol (2:1, v/v). The solids recovered from the organic solvent extract were dissolved in 5 ml of 33% (v/v) ethanol and chromatographed on a Sephadex LH-20 column (40g, 2 x 57 cm) in the same solvent. 9-ml fractions were collected at a flow rate of about 30 ml per hour. Cytokinin standards were later run on the same column, and fractions were pooled accordingly. the pooled fractions were tested for cytokinin activity in tobacco callus bioassay.

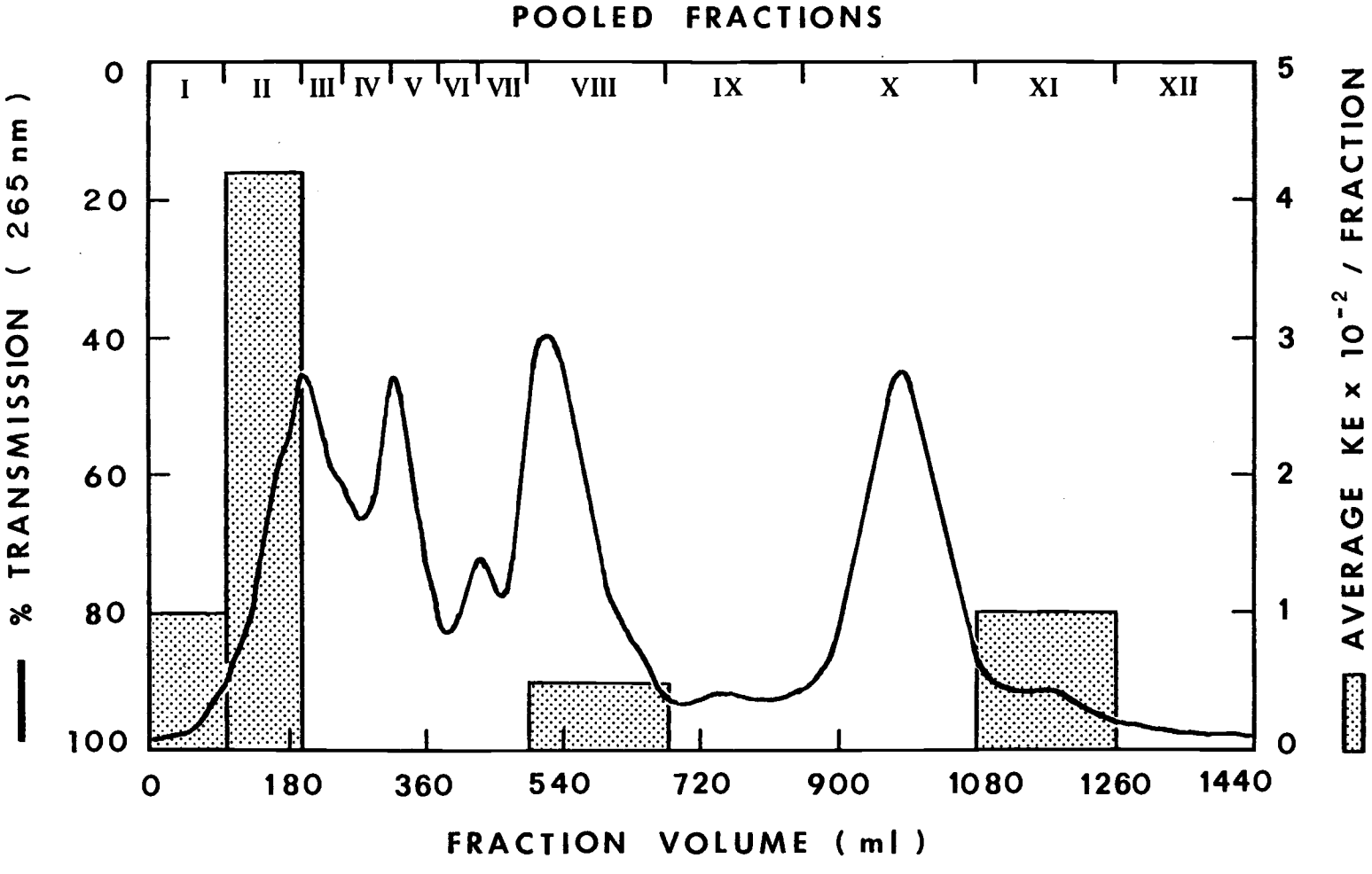

POOLED FRACTIONS
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
0
20
40
60
80
100
% TRANSMISSION (265 nm)
5
4
3
2
1
0
AVERAGE KE x 10⁻² / FRACTION
0
180
360
540
720
900
1080
1260
1440
FRACTION VOLUME (ml)

Table 13. Cytokinin activity in extracts of cytokinin autonomous callus tissue of Phaseolus lunatus cv. Kingston.

Pooled* fraction	Cytokinin standard	µg KE/kg callus tissue	
		H_2O-saturated ethyl-acetate extract	Ethyl acetate: t-butanol extract
I		—	0.03
II		—	0.11
III	io^6A	0.04	—
IV	io^6Ade	0.03	—
V	i^6A	—	—
VI		0.03	—
VII	i^6Ade	—	—
VIII		0.04	0.03
IX		—	—
X		0.03	—
XI		0.02	0.04
XII		—	—
Total		0.19	0.21

*Sephadex LH-20 column (2 x 57 cm) with 33% ethanol

6. Application of Amberlite XAD-2 procedure to the detection of cytokinin biosynthesis in tobacco callus tissue.

On the basis of the results reported above, the use of Amberlite XAD-2 appeared to hold promise of being useful in the detection of cytokinin biosynthesis from adenine-8-^{14}C. The earlier labeling studies with cytokinin-autonomous tobacco callus tissue were repeated and the effect of Amberlite XAD-2 purification on the recovery of label from tissue extracts. The results shown in Table 14 indicate that a large proportion of the label extracted from the tissue did not bind to the column. Sephadex LH-20 chromatography of the material recovered from the XAD-2 column by elution with 70% ethanol gave the profile shown in Figure 18. In contrast to the results obtained by chromatographing crude tissue extracts (Figure 14), the material recovered from the XAD-2 column gave distinct peaks of label, one of which corresponded to the elution position of i^{6}A. Rechromatography of the latter peak on Sephadex LH-20 in distilled water gave a peak of label that chromatographed with i^{6}A (Figure 19). Rechromatography of the adenine-io^{6}Ade region (Figure 20) and the adenosine-io^{6}A region (Figure 21) on Porapak Q has subsequently been attempted. This procedure is effective in separating the respective standard compounds, but no label was found associated with the elution position of either io^{6}A or io^{6}Ade.

Table 14. Effect of Amberlite XAD-2 on recovery of radioactivity in ethanol extracts of cytokinin autonomous tobacco culture tissue after incubation with ^{14}C-adenine.

Procedure	Total CPM	
	Method 1	Method 2
Crude extract (95% ethanol) (before centrifugation)	334,200	370,000
Crude extract (95% ethanol) (after centrifugation)	382,000	
Sample column-through (on XAD-2 column)		208,000
Water-washing (10 bed volumes)		203,000
Sample eluate (10 bed volumes) (70% ethanol)		54,486

Callus tissues (10.5 g and 10.2 g, respectively) were incubated for five hours with 2 μCi ^{14}C-adenine and the tissue homogenized with 25 ml of 95% cold ethanol for one min. For method 1, ethanol extract was evaporated to dryness and redissolved in 2 ml of 33% (v/v) ethanol. An appropriate amount of the sample was counted for radioactivity. After centrifugation of the sample, it was again counted. For method 2, the ethanol extract was evaporated and redissolved in 50 ml of distilled water. The aqueous solution was applied to a Amberlite XAD-2 column (0.9 x 30 cm). Samples were collected and counted for radioactivity as indicated.

Figure 18. Effect of Amberlite XAD-2 purification on the distribution of radioactivity in an ethanol extract of cytokinin autonomous tobacco callus tissue incubated with adenine-8-^{14}C for 5 hours.

Cytokinin autonomous tobacco callus tissue was incubated with 2 μCi of adenine-8-^{14}C for 5 hours as described in Materials and Methods. The tissue was homogenized in ethanol, and the ethanol extract was dried and dissolved in 50 ml of distilled water. This aqueous solution was applied to an Amberlite XAD-2 column (17 g, 0.9 x 30 cm), which was then washed with water (30 bed volumes) and eluted with 10 bed volumes of 70% (v/v) ethanol. The solids recovered from the ethanol eluate were dissolved in 2 ml of 33% (v/v) ethanol and chromatographed on the Sephadex LH-20 column (20 g, 1.5 x 50 cm) in the same solvent. Eight-ml fractions were collected at a flow rate of about 24 ml per hour. Cytokinin standards were later chromatographed on the same column. Half of each fraction was counted for radioactivity measurements.

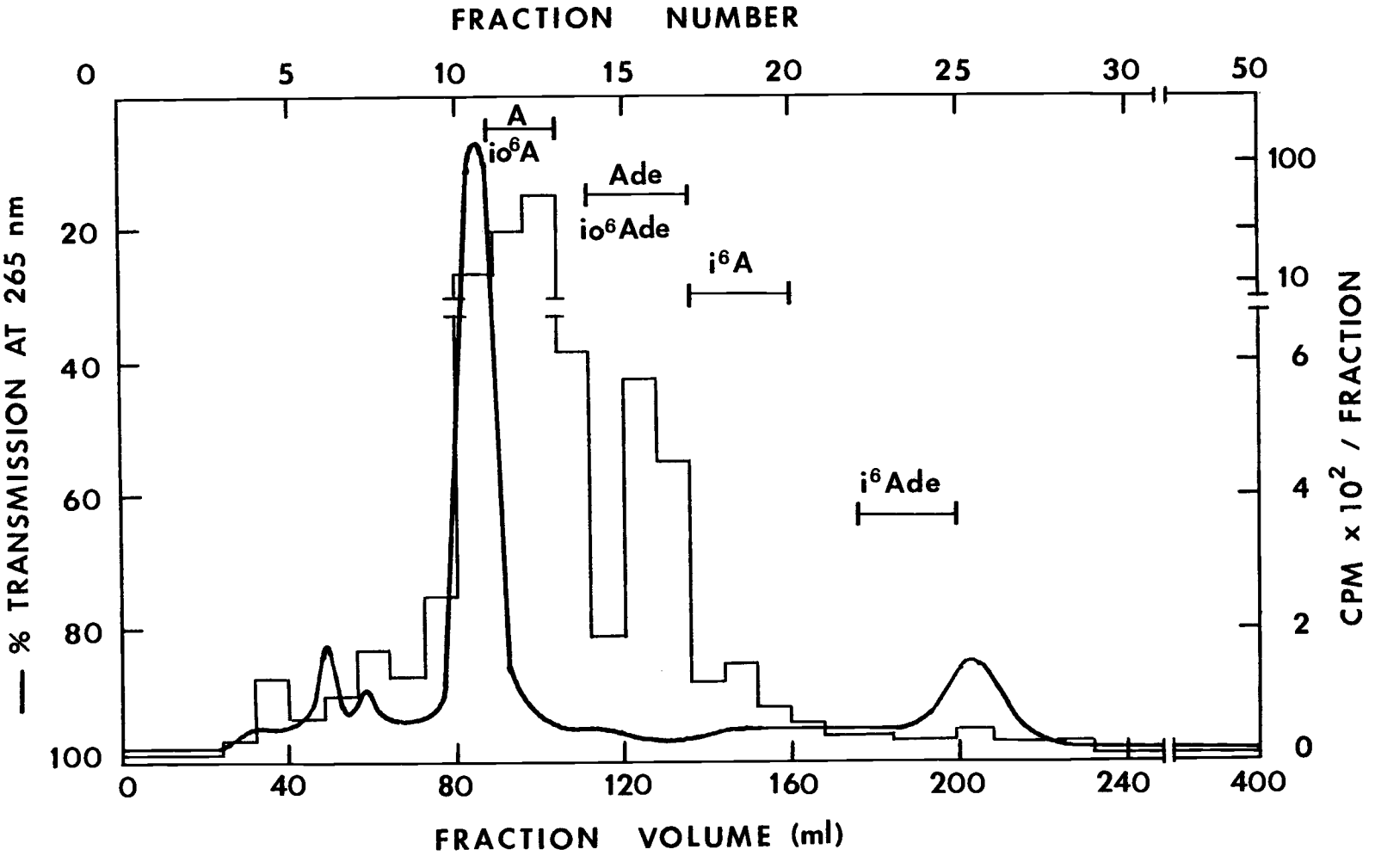

FRACTION NUMBER
0
5
10
15
20
25
30
50
A
io⁶A
Ade
io⁶Ade
i⁶A
i⁶Ade
— % TRANSMISSION AT 265 nm
20
40
60
80
100
100
10
6
4
2
0
CPM x 10² / FRACTION
0
40
80
120
160
200
240
400
FRACTION VOLUME (ml)

Figure 19. Rechromatography of the i^6A region from the Sephadex LH-20 (33% ethanol) elution profile shown in Figure 18.

Aliquots (50%) of the fractions corresponding to the eluted position of i^6A in the Sephadex LH-20 fractionation shown in Figure 18 were pooled, evaporated to dryness, and redissolved in 2 ml of water. This sample was chromatographed on a Sephadex LH-20 column (20 g, 1.5 x 52 cm) in distilled water. 8-ml fractions were collected at a flow rate of about 32 ml per hour. A i^6A was cochromatographed. Each fraction was evaporated to dryness and counted for radioactivity.

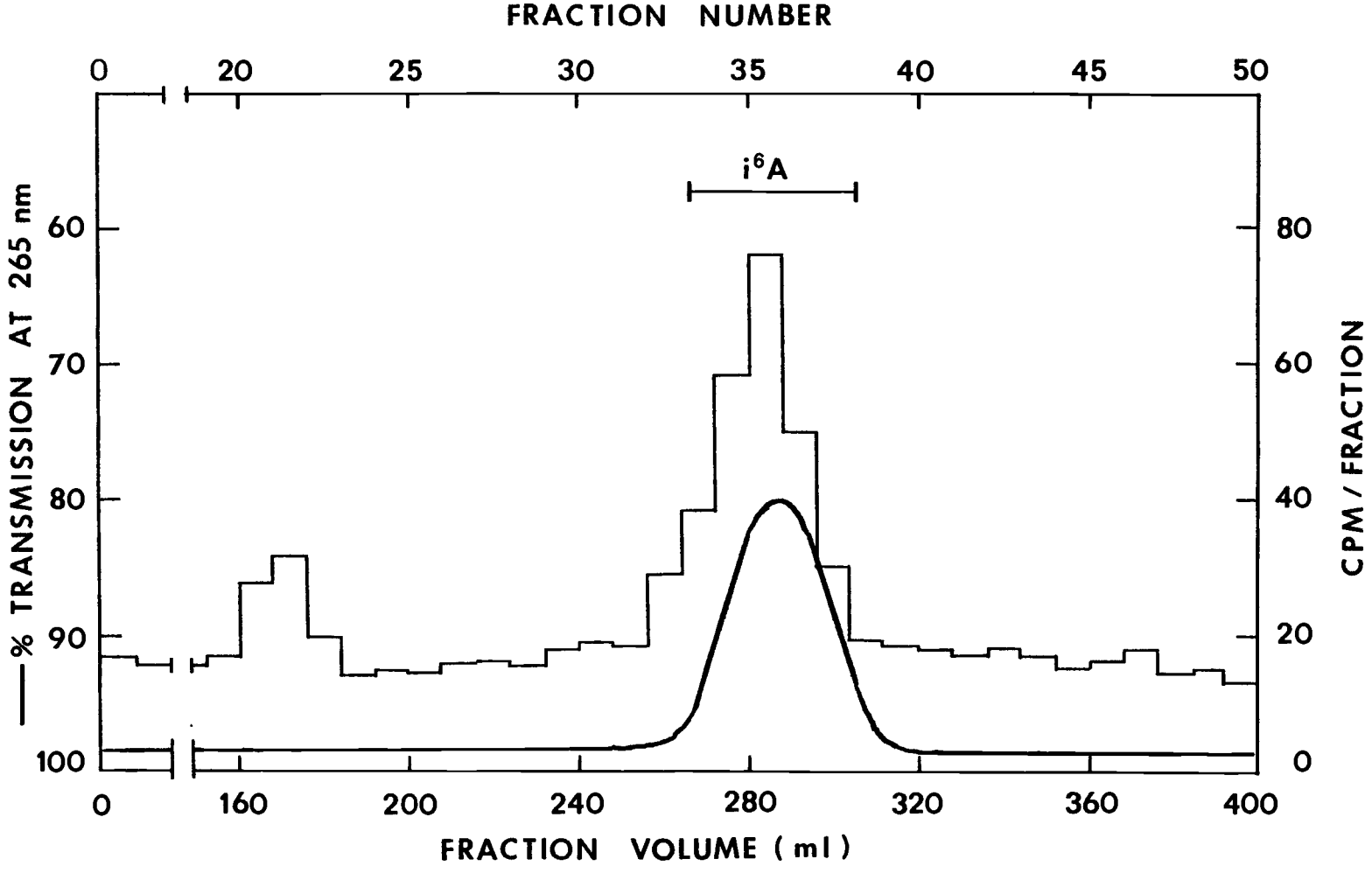

FRACTION NUMBER
0
20
25
30
35
40
45
50
i⁶A
—% TRANSMISSION AT 265 nm
60
70
80
90
100
CPM / FRACTION
80
60
40
20
0
0
160
200
240
280
320
360
400
FRACTION VOLUME (ml)

Figure 20. Rechromatography of the io^6Ade region from the Sephadex LH-20 (33% ethanol) elution profile shown in Figure 18.

Aliquots (50%) of the fractions corresponding to the elution position of io^6Ade in the Sephadex LH-20 fractionation shown in Figure 18 were pooled, evaporated to dryness, and redissolved with io^6Ade standard and adenine in one ml of 24% (v/v) ethanol, This sample was chromatographed on a Porapak Q column (5 g, 0.9 x 16 cm) in 24% ethanol. Two-ml fractions were collected at a flow rate of about 10 ml per hour. Each fraction was evaporated to dryness and counted for radioactivity.

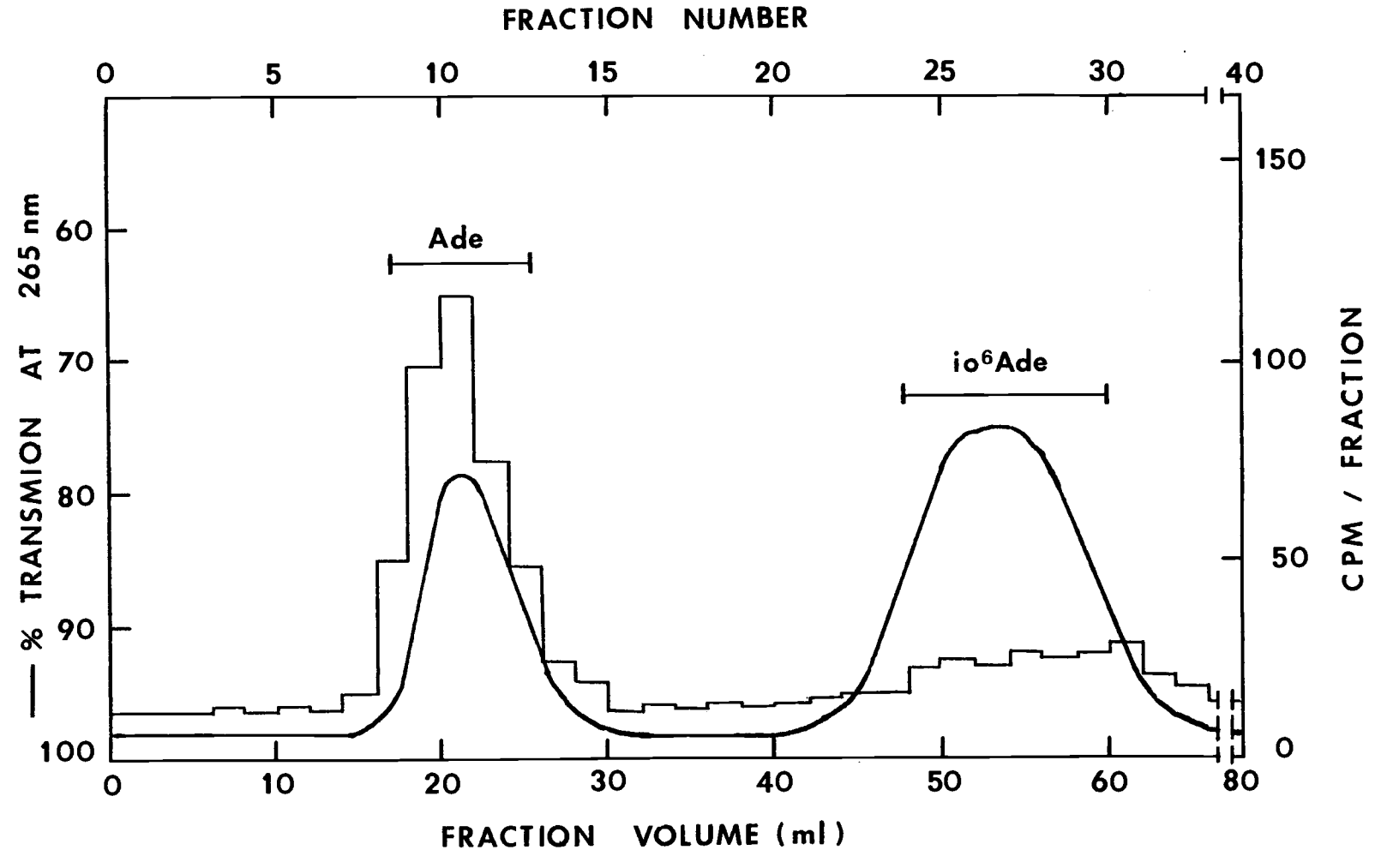
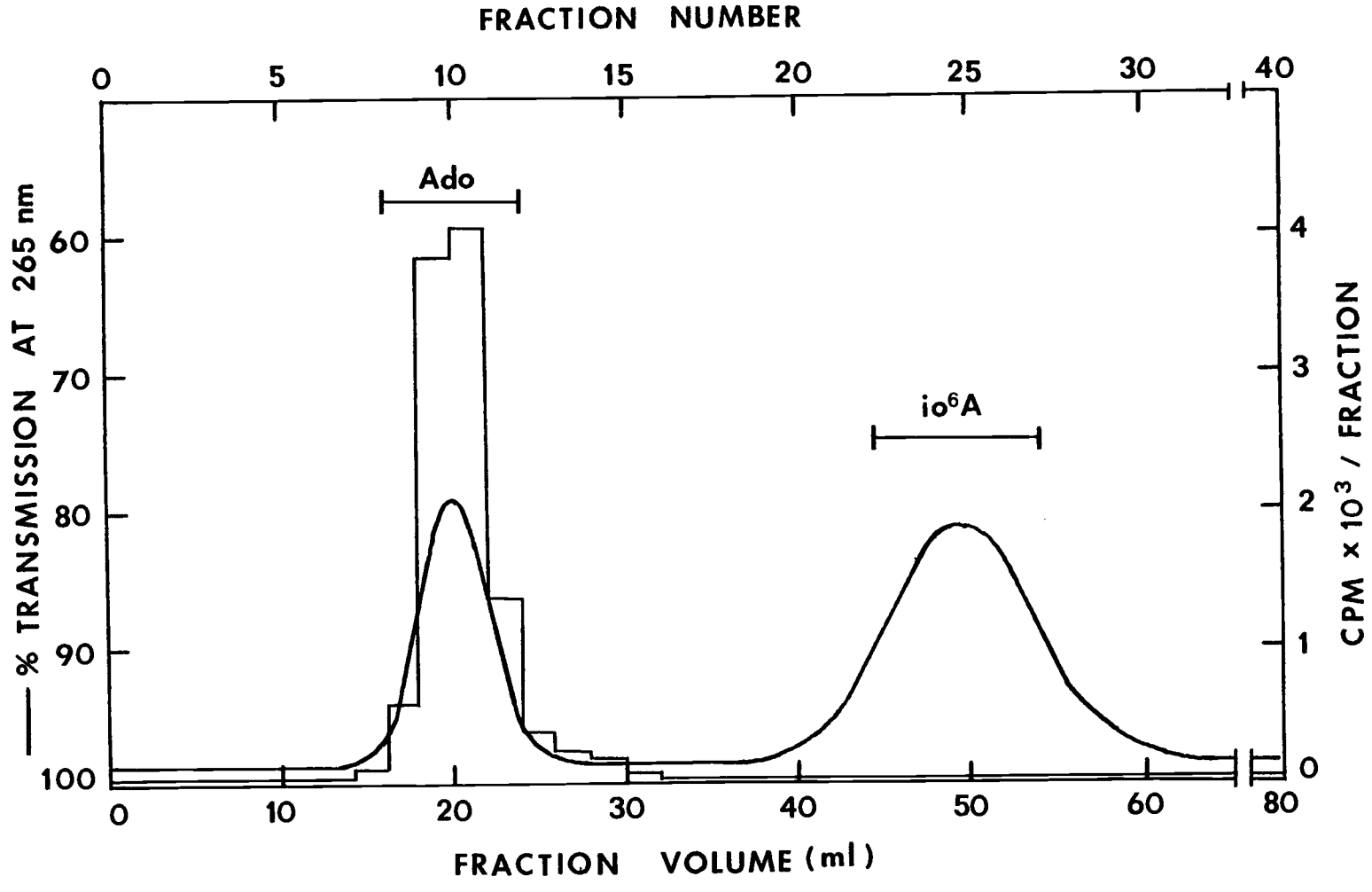

FRACTION NUMBER
0
5
10
15
20
25
30
40
Ade
io⁶Ade
— % TRANSMION AT 265 nm
60
70
80
90
100
150
100
50
0
CPM / FRACTION
0
10
20
30
40
50
60
80
FRACTION VOLUME (ml)

Figure 21. Rechromatography of the io^6A region from the Sephadex LH-20 (33% ethanol) elution profile shown in Figure 18.

Aliquots (50%) of the fractions corresponding to the elution-position of io^6A in the Sephadex LH-20 fractionation shown in Figure 18 were pooled, evaporated to dryness, and redissolved in one ml of 24% (v/v) ethanol. This sample was chromatographed on a Porapak Q column (5 g, 0.9 x 16 cm) in 24% ethanol. Two-ml fractions were collected at a flow rate of about 10 ml per hour. io^6A standard and adenosine were later chromatographed on the same column. Each fraction was evaporated to dryness and counted for radioactivity.


FRACTION NUMBER
0
5
10
15
20
25
30
40
Ado
io⁶A
— % TRANSMISSION AT 265 nm
60
70
80
90
100
CPM x 10³ / FRACTION
4
3
2
1
0
0
10
20
30
40
50
60
80
FRACTION VOLUME (ml)

V. DISCUSSION

The ability of N,N'-diphenylurea to substitute for cytokinin-active adenine derivatives in promoting the callus growth of Phaseolus lunatus genotypes has been examined in detail. In initial tests with callus tissues derived from P. lunatus genotypes cv. Kingston and P.I. 257422 (w), considerable genotypic differences in response to diphenylurea were observed. Kingston tissue required high concentrations of DPU (32 to 100 μM) for its growth and exhibited rather irregular growth in response to DPU. Callus tissue of P.I. 257422 (w) responded to low concentrations of DPU (1 μM) and exhibited vigorous and uniform growth at concentrations of 10 μM or higher. After one passage on DPU-containing medium, P. lunatus callus tissues responding to DPU were capable of continued and indefinite growth on cytokinin-free medium.

Callus tissue maintained on optimal concentration of kinetin-containing medium did not exhibit any growth on the cytokinin-free medium. These results suggested that the primary effect of DPU in these systems was to induce cytokinin autonomy.

A number of P. lunatus genotypes were screened for their ability to respond to DPU in tissue culture. In all P. lunatus genotypes tested response to DPU was associated with transformation to cytokinin-autonomous growth, but genotypic variations in ability respond to DPU were noted. Thus, P. lunatus cv. Henderson Bush, Jackson Wonder, P.I. 264239 and P.I. 257409 as well as P.I. 257422 (w) exhibited regular and vigorous growth on medium containing 32 μM DPU. Callus tissues from genotypes cv. Nema Green, P.I. 257560. P.I.180324, and

P.I. 256812 exhibited rather irregular and less vigorous growth on medium containing 32 μM DPU. Another four genotypes (P.I. 260415, 202830, 256845 and 257547) exhibiting no response or very little response to DPU have been identified.

A detailed comparison of the effects of kinetin and DPU on the growth of P. lunatus callus tissue has been done with three P. lunatus genotype (cv. Jackson Wonder, P.I. 257422-w, and P.I. 260415). The P.I. 260415 genotype differs from the other two genotypes in the sense that it failed to grow on DPU containing media. This genotype also required higher concentrations of kinetin to support callus growth than did cv. Jackson Wonder and P.I. 257422-w. Genotype P.I. 257422-w exhibited optimal response to a DPU concentration of 10 μM. A further increase in DPU concentration to 32 μM caused no further increase in fresh weight yield in this genotype. However, the maximum growth of the callus tissue on DPU was only about a third of the maximum yield obtained on kinetin-containing media. Genotype cv. Jackson Wonder was slightly less responsive to DPU than P.I. 257422 (w). In all three genotypes, the presence of DPU in the culture medium had no effect on the concentration of kinetin required to give maximum fresh weight of the callus tissue. Thus, the effects of DPU and kinetin on the growth of P. lunatus callus tissue were not additive. Transfer of callus tissue of cv. Jackson Wonder, P.I. 257422-w, and P.I. 260415 to cytokinin-free media from media containing various concentrations and combination of DPU and kinetin was used to examine the interaction of these two cytokinins in the induction of cytokinin-autonomous growth. Tissues

grown on suboptimal concentrations of kinetin and tissues grown on DPU exhibited cytokinin-autonomous growth when transferred to cytokinin-free media. Tissue grown on optimal or close to optimal kinetin concentrations remained cytokinin-dependent. At higher kinetin concentrations, the callus tissues also exhibited cytokinin-autonomous growth when transferred to cytokinin-free medium. The effect of increasing kinetin concentrations in suppressing the development of the cytokinin-autonomous growth habit was overcome to some extent by the presence of DPU in the culture media. The development of the cytokinin-autonomous growth habit in P. lunatus callus tissues exposed to suboptimal or supraoptimal concentrations of kinetin was unexpected as the tissues respond uniformly to the presence of this cytokinin in the tissue culture media.

In tests of the response of P. lunatus callus tissues to three ureidopurines, callus tissues from both P. lunatus cv. Kingston and cv. Jackson Wonder exhibited uniform and vigorous growth. At optimal concentrations, the ureidopurines gave fresh weight yield almost equivalent to that of callus tissue grown on BAP-containing medium, although all three compounds were less active than BAP. The behavior of callus tissues grown on ureidopurine-containing media was very similar to that of callus tissues grown on either BAP or N^6-cyclohexylmethylaminopurine when the tissues were transferred to cytokinin-free medium. In all cases, callus tissue grown on suboptimal cytokinin concentrations or at concentrations higher than that required to support optimal growth exhibited cytokinin-autonomous growth, and at optimal concentrations this characteristic was suppressed. It appears that the maintenance

of the cytokinin-dependent growth habit in genotypes exhibiting this response requires the presence of a critical concentration of exogenously supplied cytokinins, and only a relatively narrow concentration range is effective in suppressing cytokinin-autonomous growth. It is not clear whether the development of cytokinin autonomy in tissues grown on DPU-containing media is equivalent to the process that occurs on media containing concentrations of purine cytokinins above or below the optimum. The response of the tissues to these two types of cytokinins does not appear to be additive, and DPU promoted the development of cytokinin autonomy in tissue exposed to kinetin concentrations that by themselves would have suppressed the autonomous growth habit.

Induction of cytokinin-autonomy in cytokinin-dependent tobacco tissue culture exposed to streptomycin has been reported (Kaminek and Lustinec, 1974). Treatments of tobacco callus tissue with morphactins or aminofluorenes which are carcinogenic in animal systems have also been reported to induce cytokinin-autonomous tissues (Bednar and Linsmaier-Bednar, 1971a, 1971b). But it seems unlikely that mode of action of these compounds in inducing cytokinin autonomy in tobacco callus tissue is equivalent to that of DPU in P. lunatus callus cultures.

The behavior of tobacco tissue cultures grown on DPU contrasts with the behavior observed with P. lunatus callus tissues. Tobacco callus tissue responds uniformly to DPU and remains cytokinin-dependent even after repeated transfers on DPU-containing medium. Only a slight and irregular increase in background growth after seven transfers of tobacco callus tissue on DPU-containing medium has been observed.

The use of auxins other than indole-3-acetic acid in tobacco tissue culture medium resulted in a slight increase in growth on cytokinin-free medium, but in no case did the tissue exhibit the rapid and vigorous growth derived from P. lunatus callus tissues after exposure to DPU. The strong auxin 2,4-dichlorophenoxyacetic acid has been reported to stimulate some proliferation of soybean and tobacco callus tissue in the absence of cytokinin (Fosket and Torrey, 1969; Witham, 1968), and picloram (the auxin used for P. lunatus callus tissue) is an extremely potent auxin in most plant systems. However, the results obtained here indicate that the difference in the response of P. lunatus and tobacco callus tissue to DPU is not related to the use of different auxins in the two tissue culture media.

If the mechanism of action of DPU involves the stimulation of endogenous synthesis of purine cytokinins, the tobacco callus system provides a convenient test system. An attempt has therefore been made to develop efficient methodology for the direct detection of cytokinin biosynthesis in this system. The use of Amberlite XAD-2 columns for preliminary purification of material recovered from crude ethanol extracts of plant tissues has been tested. The use of this non-ionic resin provides mild conditions and apparently quantitative recovery of naturally occurring cytokinins as tested with commercially available cytokinins and by the recovery of cytokinins from tRNA hydrolysates and Agrobacterium culture filtrate. Amberlite XAD-2 (a cross-linked polystyrene) was preferred over Amberlite XAD-7 (an acrylic ester polymer) because of the relatively strong retention of adenine and the free base form

of cytokinins on the latter resin. Amberlite XAD-2 is a readily available material of relatively low cost and provides fast flow rates where large volumes of material must be processed. As judged by extraction of cytokinin from an autonomous strain of P. lunatus callus tissue, it should be useful as a purification step in the large scale isolation and identification of cytokinin from plant tissues and may provide an alternative to the use of Dowex 50 (Dyson and Hall, 1972; Scarbrough et al., 1973) or solvent partition method (Hemberg and Westlin, 1973; Hemberg, 1974; Scarbrough et al., 1973; Miller, 1975).

Preliminary tests of the utility of Amberlite XAD-2 in detecting cytokinin biosynthesis from labelled adenine in a cytokinin-autonomous strain of tobacco callus tissue indicate that this procedure remove sufficient background label that peaks of label incorporated into cytokinins should be easily detected. This should permit a more direct assessment of the ability of P. lunatus and tobacco callus tissues to synthesize endogenous cytokinins under a variety of conditions and should be useful in determining the nature of the effects of both DPU and purine cytokinins on the regulation of cytokinin metabolism in the tissues.

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