STUDIES ON CYTOKININ BINDING IN TOBACCO CELLS

Dissertation for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
MICHAEL RICHARD SUSSMAN
1976

This is to certify that the

thesis entitled

Studies on cytokinin binding in tobacco cells.

presented by

Michael Richard Sussman

has been accepted towards fulfillment
of the requirements for

Ph.D degree in Botany

Major professor

Date Jan 4 / 1977

ABSTRACT

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By

Michael Richard Sussman

For the detection of a small number of higher-affinity cytokinin binding sites, high specific activity N^6-benzyladenine *p*-[^{3}H]-benzyl (*p*-[^{3}H]-BA) was synthesized by a simple procedure. *p*-[^{3}H]-BA at 10 Ci/mmol was obtained by catalytic dehalogenation of *p*-bromo-N^6-benzyladenine with carrier-free tritium gas. A radiochemical purity of 97% was obtained by a single TLC purification step.

Using this *p*-[^{3}H]-BA and a particulate fraction from cytokinin-dependent tobacco cells grown in suspension culture, *high*- and *low*-affinity cytokinin binding sites were observed. The *low*-affinity binding site was stable to a heat pre-treatment and showed some specificity for cytokinin-like compounds (e.g., kinetin, 2iP and BA bind well, while adenine and the cytokinin ribosides do not). However, when the specificity was examined in finer detail with a series of chemically similar cytokinin analogues possessing different degrees of biological activity, no correlation was observed between binding and biological activities of the analogues.

In contrast to these results with the *low*-affinity binding site, the *high*-affinity binding site was heat-labile and the ability of cytokinin analogues to compete with BA correlated well with their

biological activity. Thus, on the basis of heat denaturability and binding specificity, it was concluded that only the *high*-affinity binding site is specific, and possesses the characteristics expected for a cytokinin receptor protein.

This specific binding site in a particulate reaction of tobacco cells is probably not identical to the cytokinin-binding protein isolated from wheat germ ribosomes by Fox and Erion (1975). In contrast to their findings, 1 M KCl did not solubilize the specific cytokinin binding sites.

I also observed *high*- and *low*-affinity, saturable binding sites for *p*-[^{3}H]-BA using the non-biological material, talc. However, in neither case was the binding specificity with talc similar to that observed in any cytokinin bioassay.

The analogues which were synthesized and tested for cytokinin specificity studies were the following BA derivatives possessing a single halogen atom in the N^{6} benzyl side-group: *ortho*-F-BA, *meta*-F-BA, *para*-F-BA, *ortho*-Cl-BA, *meta*-Cl-BA, *para*-Cl-BA, *ortho*-Br-BA and *para*-Br-BA.

When the biological activity of the cytokinin analogues was compared in several bioassays, an interesting difference was noted. In a tobacco cell division (suspension culture) assay, the order of biological activity was BA > *ortho*-Cl-BA > *meta*-Cl-BA = *para*-Cl-BA (inactive), but in the moss bud formation assay, the observed order of activity was *meta*-Cl-BA $\geq$ BA > *ortho*-Cl-BA = *para*-Cl-BA. Models emphasizing either steric or electronic properties of the N^{6} benzyl side-group for binding to a cytokinin receptor were proposed to explain these results in the tobacco and moss systems, respectively.

In addition, in a tobacco callus shoot formation assay, the order of activity of the analogues paralleled their activity in the cell division assay with the same tissue, though one compound, *meta*-Cl-BA, stimulated shoot formation with little or no discernible stimulation of cell division.

Efforts to determine the precise cellular location of the specific cytokinin binding site were greatly hampered by the large background of non-specific binding. Thus, for a more quantitative and detailed characterization of specific cytokinin binding sites, an alternative technique, photoaffinity labelling, was explored. A potential cytokinin photoaffinity reagent, 8-azido-N^6-benzyladenine (8-N_3-BA) was synthesized from 8-bromo-adenosine in a four-step reaction sequence with 8-azido-adenosine, 1-benzyl-8-azido-adenosine and 8-azido-N^6-benzyladenosine as intermediates.

8-N_3-BA was found to be a fully active cytokinin in the moss bud formation bioassay and slightly more active than BA in a tobacco cell division (suspension culture) bioassay. In addition, at high concentrations, where BA inhibits cell division, 8-N_3-BA did not. 8-N_3-BA was readily photolyzed by long- and short-wavelength UV light yielding product(s) which were inactive in the moss bioassay but may have had slight activity in the tobacco bioassay. These results therefore justify the future synthesis and use of radioactive 8-N_3-BA to covalently label specific cytokinin binding sites.

STUDIES ON CYTOKININ BINDING IN TOBACCO CELLS

By

Michael Richard Sussman

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology

1976

To my parents, Stella and Dan

ACKNOWLEDGEMENTS

The author wishes to express appreciation to all of his many friends in the PRL community who helped in his education, both personal and scientific. In particular, the author thanks his thesis advisor, Dr. Hans Kende, for allowing the freedom to pursue interests, for the encouragement to strive for precision in thought and writing and for his patience.

Help from the members of his guidance committee, and in particular that of Dr. Anton Lang, is acknowledged with warm appreciation.

The author wishes to thank Dr. Renata L. DeZacks for her assistance in the bioassays, and members of the mass spectrometry laboratory of Dr. C. Sweeley for all of the mass spectral analyses.

Finally, the author thanks Nancy Jo Gaedke for her loving, gentle nature and for assistance in the delivery of this thesis.

The research reported here was supported by the U.S. Atomic Energy Commission and the U.S. Energy Research and Development Administration under Contract E(11-1)-1338 and by graduate fellowships awarded to the author by the Woodrow Wilson Foundation (1971-1972) and by the National Science Foundation (1972-1975).

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LIST OF ABBREVIATIONS

BA	N^6-Benzyladenine
8-N_3-BA	8-Azido-N^6-benzyladenine
2,4-D	2,4-Dichlorophenoxyacetic acid
K_d	Equilibrium dissociation constant
R_t	Total concentration of binding sites
TLC	Thin-layer chromatography
GLC	Gas-liquid chromatography
UV	Ultraviolet light
Ci	Curie
S.E.	Standard error of the mean; calculated according to the equation: standard error = standard deviation/$\sqrt{n}$, where n = number of replicate samples

GENERAL INTRODUCTION

Despite much research and speculation, we still have no knowledge of the early (0-5 min) biochemical reactions which occur in a plant cell in response to the application of a hormone.

One approach to this problem is to identify and localize the cell component(s) which binds the hormone and initiates the biochemical reactions leading to the physiological response. This cell component is commonly referred to as the hormone "receptor" and, in all cases known to date, has been found to be a protein the binding activity of which, like that of enzymes, is sensitive to denaturants which destroy protein conformation. For example, protein receptors have been isolated which bind to and mediate the effects of the following animal hormones and drugs: steroids (Jensen and DeSombre, 1972), peptide hormones (Cuatrecasas and Hollenberg, 1976), acetylcholine (Hall, 1972), opium (Sharma et al., 1975), and LSD (Nathanson and Greengard, 1974). In bacterial systems, analogous effector-receptor interactions occur, for which functional binding proteins have been isolated. Such proteins include: plasma membrane-bound chemotactic receptors (Spudich and Koshland, 1975), a soluble cAMP receptor (Zubay et al., 1970), and the numerous transcriptional repressors (cf. Riggs and Bourgeois, 1968).

In addition to the precedents mentioned above, a theoretical argument can be made as to why hormone and drug receptors must be proteins. It is generally accepted that only proteins are capable of

forming the wide diversity of specific and *high*-affinity binding sites which are required to explain these common characteristics of hormone and drug responses: (1) stereospecificity of hormones and drugs and (2) the very low concentrations at which the responses are elicited and also saturated.

With regard to plant hormones, the above theoretical argument is equally valid and has been critically discussed (Kende and Gardner, 1976). Though no receptor protein for a plant hormone has yet been isolated (Kende and Gardner, 1976), pioneering work in this field by Hertel and his co-workers has resulted in the identification of a specific binding site for the auxin transport inhibitors, 1-N-naphthylphthalamic acid (Thomson, 1972; Thomson et al., 1973) and morphactins (Thomson and Leopold, 1974), which appears to be localized in the plasmalemma. In addition, the interest which their studies on the specific binding of auxin to membrane fractions (Hertel et al., 1972; Hertel, 1974; Dohrmann, 1975) has generated, as evidenced by the increasing number of reports from other laboratories (Oostrom et al., 1975; Batt et al., 1976; Batt and Venis, 1976; Dollstadt et al., 1976; Kasamo and Yamaki, 1976) suggests that a breakthrough in this field may be near.

The main object of the following study has been to detect and characterize *in vitro* the cytokinin binding protein(s) which may act *in vivo* as the receptor(s) for this class of plant hormones.

All active cytokinins are adenine derivatives substituted at the N^6 position with a hydrophobic side-group (see Leonard, 1974, for the most recent review on cytokinin chemistry). *Cis*- and *trans*-N^6-(4-hydroxy-3-methylbut-2-enyl)adenine (*cis*- and *trans*-zeatin) and N^6-(3-methylbut-2-enyl)adenine (2iP) are the most abundant natural

cytokinins, while N^6-furfuryladenine (kinetin) and N^6-benzyladenine (BA) are the most commonly used synthetic ones. These compounds were discovered on the basis of their remarkable stimulation of cell division in cultured plant cells (Miller et al., 1955). They are now known to effect many diverse physiological processes, including bud formation in mosses, shoot formation in tobacco callus, and senescence and directed transport of assimilates in higher plants (Kende, 1971).

The discovery that cytokinins were located adjacent to the 3' end of the anticodon of certain tRNA's led to much speculation as to whether this was causally related to the biological activity of this plant hormone. Kende and Tavares (1968) were the first to provide evidence that this was not so, and their conclusion has since been confirmed by many others (this evidence is summarized by Kende and Gardner, 1976).

Recently, Hecht, Skoog and their co-workers have described an exhaustive and systematic study comparing the biological activities of purine substituted and deaza analogues of cytokinins in a tobacco callus bioassay. The results of their experiments, in which the likely sites of metabolism of the purine ring of cytokinins have been modified to preclude such metabolism, has led to the conclusion that metabolic modification is not a prerequisite for cytokinin activity, "i.e., that the active form of the cytokinins can be the exogenous species themselves" (Hecht et al., 1975). These studies suggest, but do not prove, that a non-covalent rather than covalent interaction of the cytokinin molecule with some cell constituent(s) is required for the biological response.

Direct, but preliminary, evidence for the non-covalent binding of cytokinins to a receptor has been obtained by autoradiography using ^{14}C-BA in moss protonemata (Brandes and Kende, 1968). In addition, results on cytokinin binding to plant ribosomes have been reported from two laboratories (Berridge et al., 1970; Fox and Erion, 1975). Though there was no effect of cytokinins on *in vitro* protein synthesis (Berridge et al., 1972), *in vitro* inhibition of a ribosomal protein kinase was observed (Ralph et al., 1972). This result was confirmed by Keates and Trewavas (1974), but was interpreted by these authors as a non-physiological effect of high cytokinin concentrations due to competition with ATP at the substrate binding site. This conclusion has since been refuted (Ralph et al., 1976). Takegami and Yoshida (1975) have also recently described a soluble cytokinin binding protein from tobacco leaves.

Unfortunately, it is not possible to correctly evaluate the physiological significance of the cytokinin binding observed in these studies since no adequate tests of specificity have been performed. As will be shown in the present study (Section III), there are non-specific saturable binding sites for cytokinins, even in non-biological material such as talc, which superficially mimic specific binding sites. As was discussed in greater detail by Kende and Gardner (1976), in order to assign physiological significance to a binding site, it is essential that the specificity of binding be critically tested with analogues that are chemically very similar but have widely diverging degrees of biological activity.

In the present study, my goal has been to differentiate between specific, *high*-affinity and non-specific, *low*-affinity cytokinin binding sites in homogenates of tobacco cells. The report of this

study is divided into four sections. In Section I, I describe the chemical synthesis of a high specific activity, tritiated cytokinin. This compound was deemed necessary for the detection of specific, *high*-affinity binding sites which are present at low concentrations in the plant cell. In Section II, the biological activities of a series of cytokinin analogues are given; these data are needed to relate the *in vitro* binding results to the *in vivo* physiological responses.

In preliminary experiments with ^{14}C-BA, a large amount of non-specific binding to the particulate fraction of tobacco cells was observed. Therefore, the emphasis for designing binding experiments with *p*-[^{3}H]-BA (Section III) was placed on deciding with confidence whether a specific binding site was present in the particulate fraction of tobacco cells. In order to allow in future studies a more detailed characterization of the observed, specific cytokinin binding site, I have described in Section IV the synthesis and biological activity of a cytokinin photo-affinity reagent, 8-azido-benzyladenine.

SECTION I

THE SYNTHESIS OF A RADIOACTIVE CYTOKININ WITH HIGH SPECIFIC ACTIVITY

Introduction

Receptors for animal hormones (Cuatrecasas et al., 1974) and drugs (Goldstein, 1974; Farrow and Vunakis, 1972; Nathanson and Greengard, 1974) are present in tissues at low concentrations, and the binding of ligands to these receptors is more readily detected if a ligand of high specific radioactivity is available. This is especially true if one wishes to detect a small number of higher-affinity binding sites in the presence of a large excess of lower-affinity sites. Ligands labelled with ^{14}C cannot be obtained at sufficiently high specific activity, hence most studies employ tritiated or iodinated ligands.

Previously published attempts to introduce a stable tritium label into biologically active cytokinins by the Wilzbach method or by catalytic exchange in an aqueous solution (Berridge et al., 1970; Letham and Young, 1971; Elliott and Murray, 1972; Walker et al., 1974) have failed to produce a specific radioactivity greater than 231 mCi/mmol. In this section, I describe a new and simple method for synthesizing a radioactive cytokinin, N^6-benzyladenine[*p*-^{3}H-benzyl] (*p*-^{3}H-benzyl) with tritium in a stable, known position at near maximum specific radioactivity. The method employs catalytic dehalogenation (hydrogenation) of a halogenated precursor, *p*-bromo-N^6-benzylaminopurine (*p*-Br-BA).

The procedure used to synthesize *p*-Br-BA was that of Elion et al. (1952) as described by Okumura et al. (1959). Though the synthesis of the *o*-, *m*-, and *p*-Cl analogues of 6-benzylaminopurine (BA) had already been reported (Okumura et al., 1959), we chose to synthesize the previously unreported Br analogues since exchange with tritium is considered easier with this halogen. The *p*-position was chosen to reduce any possibilities of steric hindrance in the synthesis and dehalogenation reactions.

A condensed report of this work has appeared (Sussman and Firn, 1976).

Materials and Methods

Synthesis of *p*-Br-BA

6-(Methylmercapto)purine (0.3 g) was added to *p*-bromo-benzylamine (1.25 g) and heated at 120-130°C in a closed tube for 18 hr with intermittent agitation. *p*-Br-BA was precipitated by the addition of methylene chloride and collected by filtration on Whatman GF/C filter paper, followed by washing with methylene chloride. After recrystallization from dimethylformamide, the product was collected by filtration, washed with methylene chloride and dried in an oven at 80°C overnight. The yield was 28%. In a separate experiment, recrystallization from ethanol gave a similar yield.

A preliminary Beilstein test of the recrystallized product indicated the presence of halogen, which was confirmed by obtaining a green color after heating a sample on a copper wire over a gas flame. The irregularly shaped crystals changed to needles before melting at 288-289°C. Elemental analysis found: C, 47.34; H, 3.38; N, 22.96; Br, 26.34%. $C_{12}H_{10}N_5Br$ requires: C, 47.39; H, 3.31;

N, 23.03; Br, 26.27%. Identity of the compound was further confirmed by its UV, IR and mass spectra.

The UV spectrum is indistinguishable from BA, with the following maxima at acid, neutral and basic pH: $\lambda_{max}^{50\%\ EtOH}$nm(log Σ): pH 2, 275.5 (4.26); pH 7, 269.5 (4.28); pH 12, 275.5 (4.28) with a shoulder at 280-285 nm.

The IR spectrum (in KBr discs) is also similar to BA in showing (1) an unsubstituted purine N-9 position (typical weak absorption peaks at 2400-2800 cm^{-1}), and (2) the absence of the strong primary amine N-H stretching absorption peak at 3380 cm^{-1} seen in *p*-bromobenzylamine. Finally, the assigned structure was confirmed by mass spectrometry (Figure 1). The natural abundance of the two stable isotopes of Br, Br^{79} and Br^{81}, in the ratio 100:97.5, permits facile identification of Br-containing fragments. The fragmentation pattern of *p*-Br-BA is similar to that of BA. The only peaks showing the presence of Br are the molecular ion, at *m/e* 303, 305 and fragments at *m/e* 169, 171 and 184, 186 identified respectively as the Br substituted tropylium ion ($C_7H_5Br^+$; analogous to *m/e* 91 in BA) and a species ($C_6H_5BrCH_2N^+H$; *m/e* 106 in BA) formed by the loss of the purine ring system. In addition, intense peaks are observed at *m/e* 89 and *m/e* 90 corresponding to the loss of HBr and Br, respectively, from the tropylium ion. All other major peaks were accounted for as arising from the purine ring.

The purity of *p*-Br-BA was checked by TLC on silica gel, cellulose and aluminum oxide (Table 1). In all instances, only one compound was detected, using several visualization techniques. In particular, the absence of potentially reducible unreacted *p*-bromobenzylamine was verified by the absence of ninhydrin positive spots in the above TLC

Figure 1. Mass spectra of benzyladenine (BA) and *para*-bromobenzyladenine (*p*-Br-BA). Spectra were obtained by direct probe at 70 eV using an LKB MOdel 9000 Mass Spectrometer.

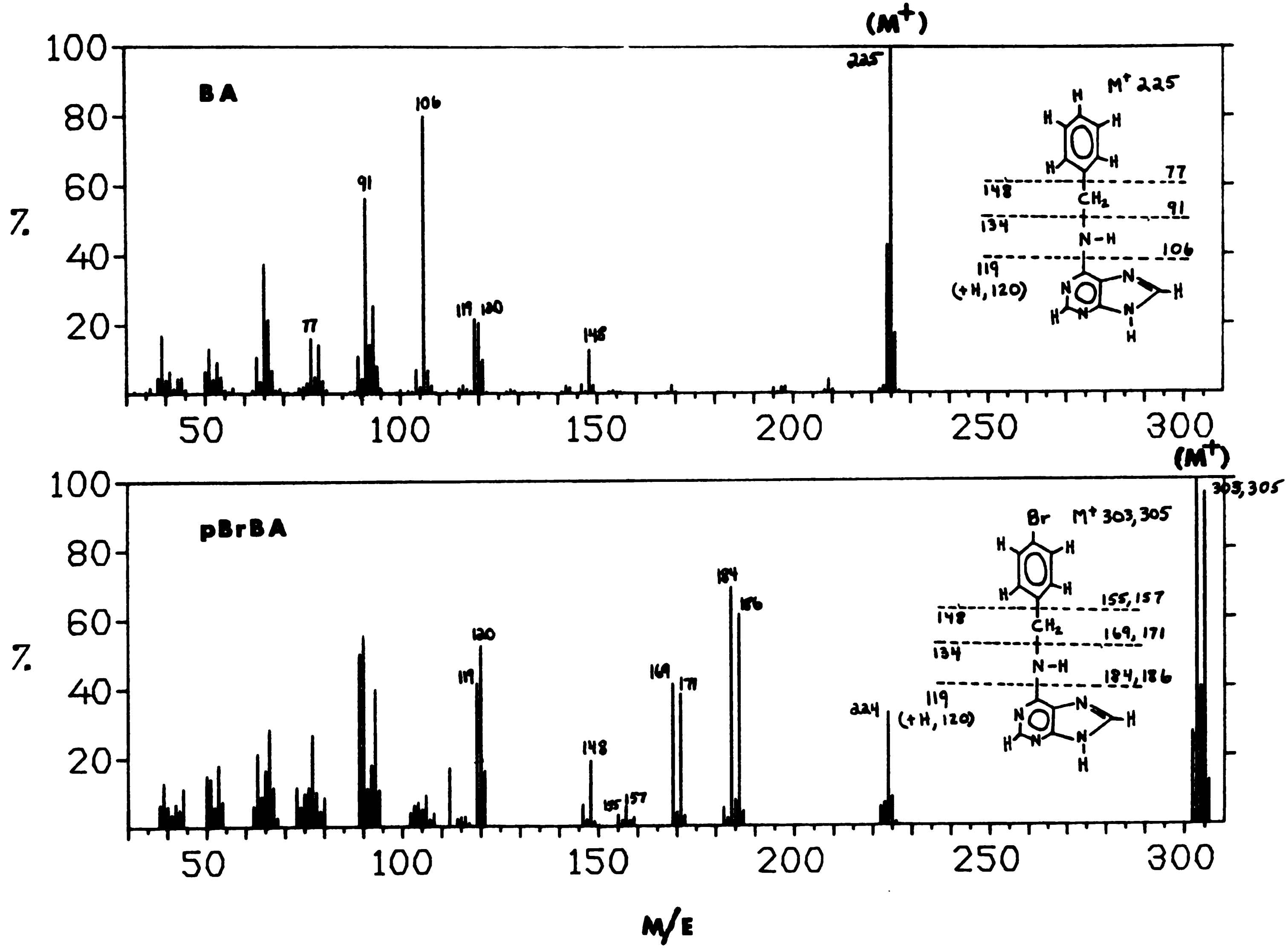

(M+)
225
BA
M+ 225
106
91
77
119 120
148
134
119
(+H, 120)
(M+)
303,305
pBrBA
Br M+ 303,305
155,157
169, 171
184,186
120
119
169
171
184
186
224
155 157
M/E
%

Figure 1

Table 1. R_f values of compounds used in the synthesis of *p*-[^{3}H]-BA in different thin layer chromatography systems

Compound	TLC System		
	Silica gel[a]	Cellulose[b,c]	Aluminum oxide[d]
BA[1,2]	0.36	0.64	0.72
p-Br-BA[1,2,5]	0.36	0.54	0.72
6-(Methylmercapto)purine[1,3]	0.36	0.44	-
p-Bromobenzylamine[4]	0.09	0.50	-
Benzylamine[4]	0.07	0.80	-

Visualization methods: [1]quenching of fluorescence induced by excitation at 254 nm after spraying with 0.1% fluorescein in ethanol; [2]purple fluorescence after excitation at 254 nm; [3]yellow fluorescence after excitation at 254 nm; [4]ninhydrin stain; [5]silver chromate stain for purines (Whitfield, 1969) using commercial plastic-backed silica gel and cellulose TLC plates.

Solvent systems: [a]10% methanol in methylene chloride; [b]0.01 N HCl; [c]cellulose plates were pre-washed with 0.1 N HCl and dried just prior to use; [d]H_2O saturated ethyl acetate.

systems. Absence of impurities was also demonstrated by GLC (Figure 2) on a 3% SE-30 column in which only a single peak was observed. As expected from comparison with the effect of bromine substitution on the retention time of benzylamine derivatives, *p*-Br-BA had a retention time 2-3 times that of BA.

Dehalogenation reaction with $^1H_2$ and $^3H_2$—

The dehalogenization reaction with $^1H_2$ gas was performed by us on the same small scale as the later reaction with $^3H_2$ gas, in order to allow more direct comparison between the two reactions. This entire procedure was performed at room temperature. Ten percent palladium on carbon (3 mg) was added to dimethylformamide (2 ml) containing 10 µl triethylamine in a 5 ml reaction vessel containing a Viton stopper and a Teflon magnetic stirring bar. The vessel was evacuated, $^1H_2$ gas added and kept at a slight pressure (approximately 0.7 kg/cm^2 above atmospheric pressure) for 30 min to saturate the Pd. The vial was then opened, *p*-Br-BA (10 mg) added and allowed to dissolve by stirring for 3 min. The vessel was closed, evacuated and $^1H_2$ applied again at slight pressure for the time denoted. For measuring the time course of the dehalogenation reaction by GLC, 50 µl samples were withdrawn periodically, filtered to remove charcoal and silylated just prior to injection. The time course (Figure 2) showed that after 1 hr less than 10% of the original *p*-Br-BA was left, and the reaction was essentially completed after 2 hr. This conclusion was confirmed by the absence of a *p*-Br-BA zone in TLC on cellulose of the 2-hr reaction product. The identity of the product was verified by co-chromatography with authentic BA on cellulose TLC (Table 1), GLC and by GLC-MS (Figure 3).

Figure 2. Time course of dehalogenation reaction of *p*-Br-BA with non-radioactive H_2 gas, as measured by GLC. Samples were silylated in dimethylformamide by adding a 3- to 5-fold excess volume of N,O-bis-(trimethylsilyl)-trifluoroacetamide containing 1% trimethylchlorosilane. GLC was performed using a 1.8 m x 1.83 mm glass column packed with 3% SE-30; column temperature, 205°C isothermal, injection port temperature, 225°C, flame detector temperature, 250°C, carrier gas He at 87.5 ml/min.

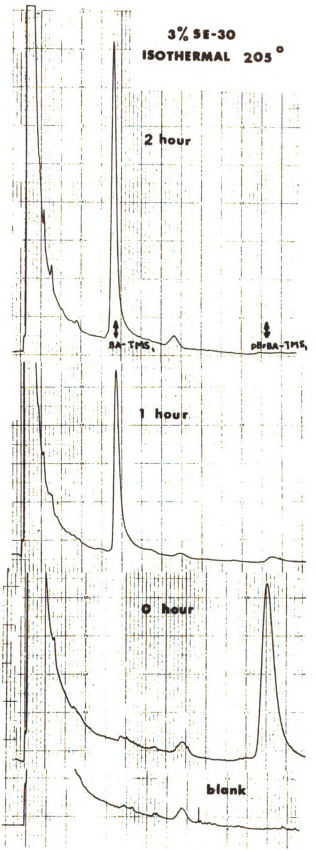

3% SE-30
ISOTHERMAL 205°
2 hour
BA-TMS
pHBA-TMS
1 hour
0 hour
blank

Figure 2

Figure 3. Mass spectrum after GLC of the trimethylsilyl derivative of the dehalogenation product with *p*-Br-BA. The retention time and spectrum obtained are identical to that observed with the authentic trimethylsilyl derivative of BA. Silylation conditions were as in Figure 2. GLC-MS was performed using a LKB Model 9000 Mass Spectrometer at 70 eV and a 1.8 m x 1.83 mm glass column packed with 2.5% SP-2401 with the carrier gas at 30 ml/min. The column temperature was programmed at 4°C/min and the retention times observed were 5 min for BA-TMS_1 and 12 min for *p*-Br-BA-TMS_1.

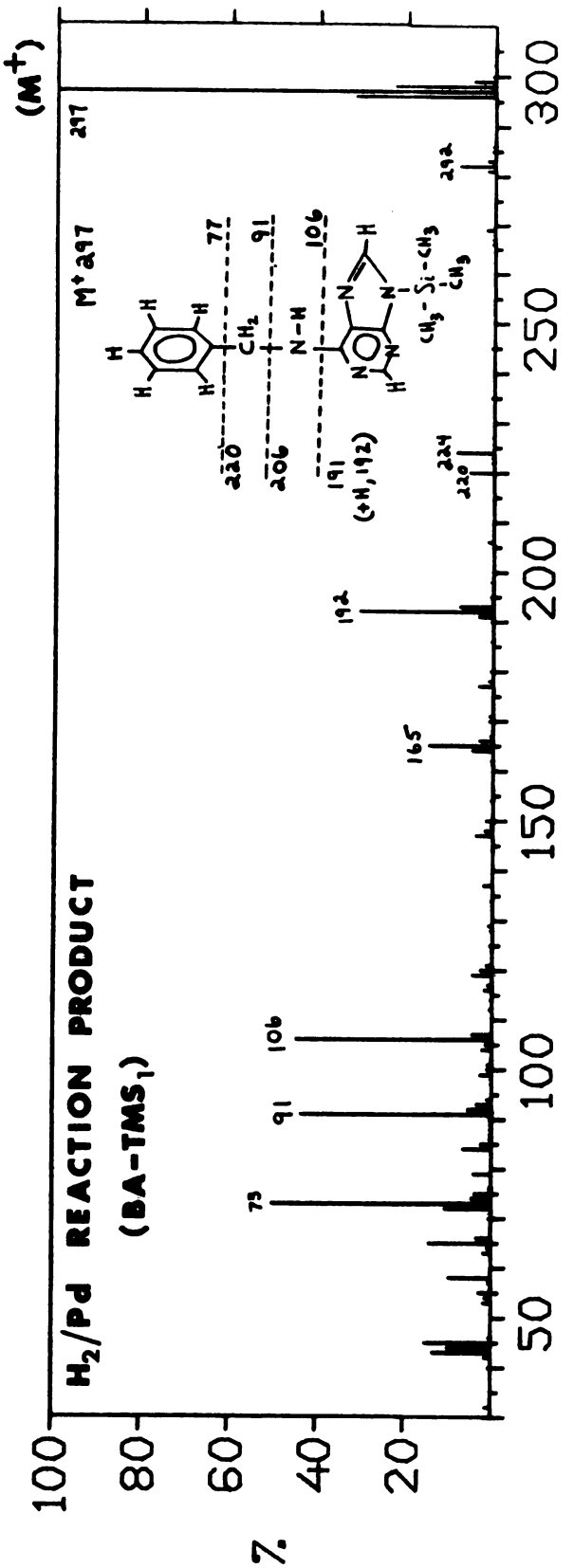

H2/Pd REACTION PRODUCT
(BA-TMS1)
(M+)
297
M+297
292
77
91
106
220
206
191
(+H,192)
224
220
192
165
73
91
106
%
M/E
100
80
60
40
20
50
100
150
200
250
300

Figure 3

For dehalogenation with $^3H_2$ gas, a sample (10 mg) of *p*-Br-BA was sent to Mallinkrodt Chemical Works (Box 5439, St. Louis, MO 63160) with instructions to perform the reaction exactly like the reaction with non-radioactive H_2 gas but with $^3H_2$ at maximum specific activity (59 Ci/mmol). Volatile ^3H was removed from the radioactive product, and the sample was purified on preparative cellulose TLC as described for the non-radioactive reaction. Again, only a single fluorescent zone at the R_f of BA was observed. The sample was eluted from the cellulose with 50% ethanol and subsequently stored in this solvent at -80°C at a concentration of 3-30 x 10^9 dpm/ml. The chemical concentration of this sample was measured by UV absorbance at 270 nm and by comparison with the absorbance of standard authentic ^1H-BA solutions. The specific radioactivity was calculated to be 10 Ci/mmol. The UV spectrum for the radioactive sample was found to be identical to BA at acid, neutral and basic pH. In addition, the radioactivity co-chromatographed with BA in 3 TLC systems and on Sephadex LH-20 column chromatography, as described below.

Purity and stability of *p*-[^3H]-BA

The radiochemical purity of this compound was shown to be at least 97% by chromatography using three TLC systems (Figure 4) and by column chromatography on Sephadex LH-20 (Figure 5). Re-chromatography on silica gel TLC raised this value to 99%. With some commercial silica gel plates (Merck pre-coated glass TLC plates), 5-10% of the radioactivity stayed at the origin. Since re-chromatography of the BA zone in the same system gave the same percentage of non-moving radioactivity, and since it was not observed on other silica gel TLC preparations (Brinkman Polygram Sil G pre-coated plastic sheets), it was considered

Figure 4. Thin-layer chromatography of *p*-[^{3}H]-BA in three systems.

THIN LAYER CHROMATOGRAPHY of p-[^{3}H]-BA

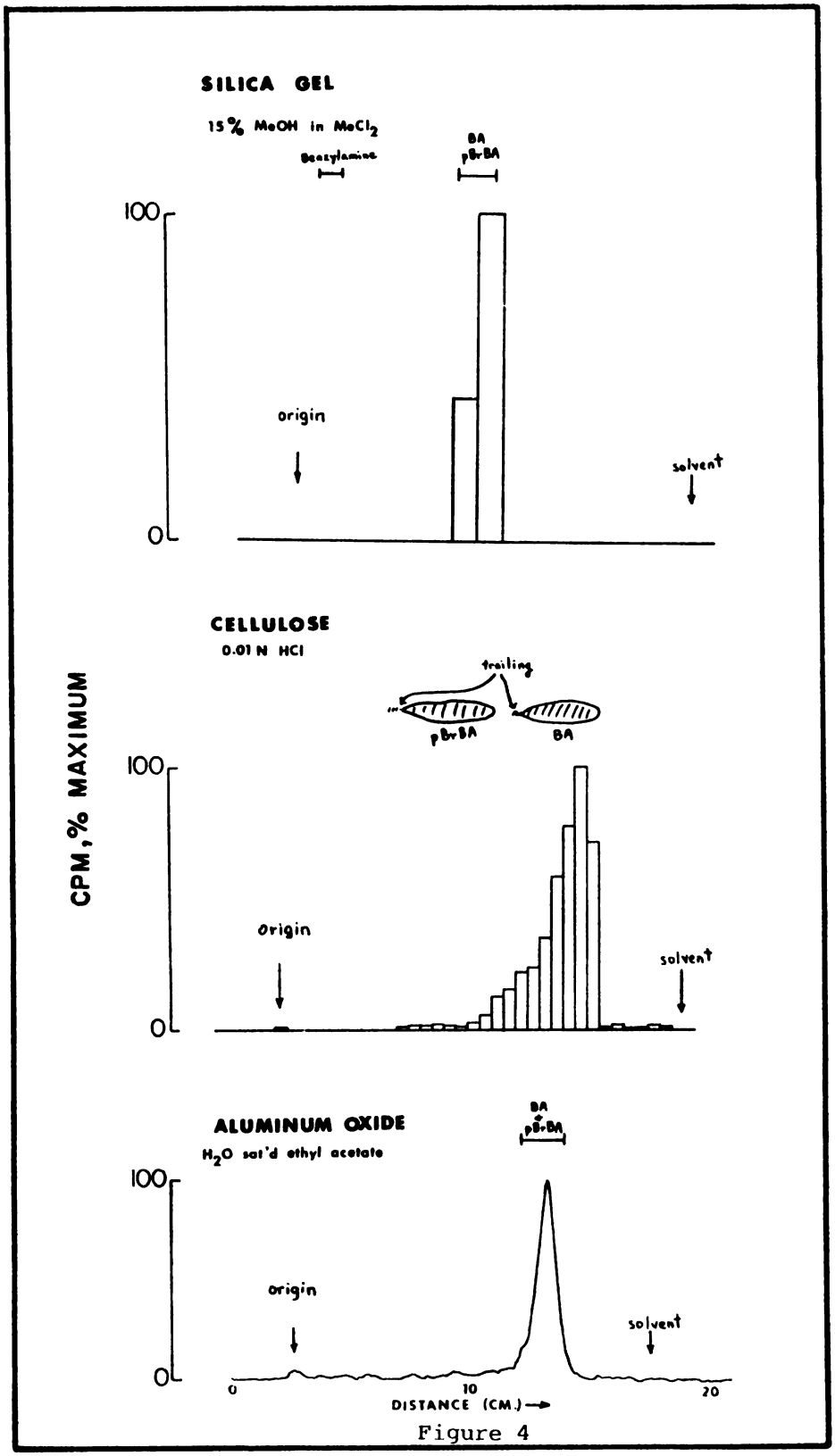


Figure 4

Figure 5. Chromatography of BA, *p*-Br-BA and *p*-[^{3}H]-BA on Sephadex LH-20. A 2.2 cm (i.d.) glass column containing 27 cm bed height of Sephadex LH-20 (25 g dry) was used. The running solvent was 95% ethanol at a flow rate of 52 ml/hr and 6.3 ml fractions were collected. In A, 1.4 ml of 0.6 mM carrier-free *p*-[^{3}H]-BA in 95% ethanol was chromatographed alone. In B, 2 ml of 95% ethanol containing 4 mg BA and 2.4 mg *p*-Br-BA was spiked with a small amount of *p*-[^{3}H]-BA (5×10^6 cpm) and chromatographed.

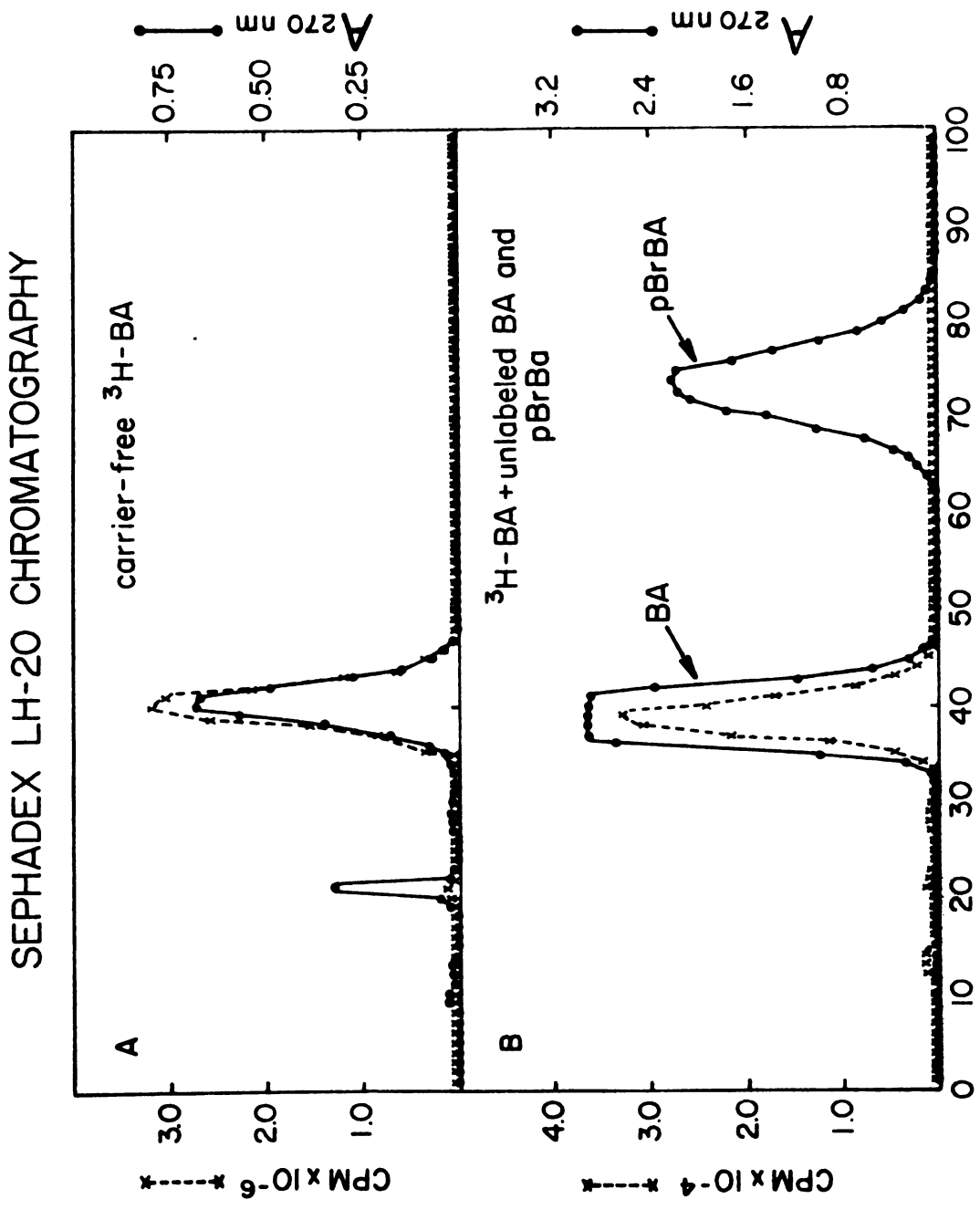

SEPHADEX LH-20 CHROMATOGRAPHY
A
carrier-free ^{3}H-BA
B
^{3}H-BA + unlabeled BA and pBrBa
BA
pBrBA
A 270 nm
CPM x 10-6
CPM x 10-4
FRACTION

Figure 5

to be an artifact produced by that particular silica gel preparation. Because the trailing of both BA and *p*-Br-BA on cellulose TLC did not permit their complete resolution and a 5-10% contamination with radioactive or non-radioactive *p*-Br-BA might therefore have gone undetected, another chromatographic system was desired. Since the BA sample is of such high specific activity, GLC, which gives complete separation of BA and *p*-Br-BA, could not be safely used to quantitate a small amount of non-radioactive *p*-Br-BA present in the tritiated sample. Chromatography on Sephadex LH-20 was found to give excellent separation of BA and *p*-Br-BA (Figure 5B). When carrier-free *p*-[^{3}H]-BA was chromatographed (Figure 5A), I observed only one major peak of radioactivity and absorbance, along with a smaller spike of a non-radioactive contaminant, proving the complete absence of *p*-Br-BA in this sample. The percent of the total radioactivity and $A_{270\ nm}$ migrating as BA in this sample was 91 and 85%, respectively. This sample, which had been stored for 13 months after the initial cellulose TLC purification, likewise showed 5-10% radioactive impurities on silica gel TLC which were not present prior to the storage period. Finally, constant specific activity (in this experiment, 9 Ci/mmol) through the BA zone was observed, demonstrating further the purity of *p*-[^{3}H]-BA obtained by this procedure.

The amount of volatile tritium in the sample purified by cellulose TLC was calculated to be less than 2-3%. After autoclaving for 20 min in aqueous buffers at pH 2, pH 7.5 and pH 10, this value rose to 10, 18 and 15%, respectively. Under similar conditions, other workers (Evans et al., 1970) have reported for adenine-2-[^{3}H], values of 2% at pH 1, 42% at pH 9-10 and for adenine-8-[^{3}H], 5% at pH 1, 53% at pH 9-10. Since the exchange of ^{3}H in purine-labelled adenine

is considered alkali mediated (Shelton and Clark, 1967), this experiment indicates that ^{3}H in *p*-[^{3}H]-BA prepared by this dehalogenation procedure is located in a less labile position.

Discussion

The technique described is a very easy procedure for obtaining a potent cytokinin radioactively labelled in a specific, known position at a high specific activity and radiochemical purity. The label appears to be quite stable, with less than 5-10% radiochemical decay detected after a 13-month storage of a concentrated sample at -18°C in 50% ethanol. Other workers may wish to substitute Sephadex LH-20 chromatography for cellulose TLC in the purification of the dehalogenation reaction product since this Sephadex chromatography was found to give an excellent separation of BA and *p*-Br-BA and since it is a convenient way of handling large amounts of radioactivity. In addition, the chromatographic solvent (35% ethanol) is suitable for storing the labelled cytokinin.

Caution should be exercised in work with radioactive benzyladenine at the high specific radioactivity described herein. We observed the binding of radioactivity in an aqueous solution to the walls of laboratory glassware. This binding can be reduced by the simultaneous presence of a low concentration (5×10^{-7} M) of non-radioactive BA in a manner similar to that described for the binding of insulin to silica and other non-biological material (Cuatrecasas and Hollenberg, 1975a). We have quantitated this phenomenon with *p*-[^{3}H]-BA by measuring its binding to talc, as will be discussed in Section III.

SECTION II

BIOLOGICAL ACTIVITY OF CYTOKININ ANALOGUES

Introduction

A major problem in hormone receptor binding studies is the assessment of the physiological relevance of the observed binding. In many cases there is great difficulty in distinguishing artifactual, non-specific binding from binding which is specific, i.e., is required to initiate the *in situ* physiological response to that hormone (Cuatrecasas et al., 1975b; Cuatrecasas and Hollenberg, 1976). Among the criteria available to assess binding specificity is the correlation of structure-activity relationships obtained with hormone analogues in the biological response, with that obtained in the binding assay.

Ideally, the analogues to be compared should have very similar chemical properties, but widely divergent biological activity. Clearly, a comparison of benzyladenine or kinetin (representing active cytokinins) with adenine or adenosine (representing inactive cytokinins), as was done by Takegami and Yoshida (1975), is not a sufficiently critical test of binding specificity, since these two classes of compounds are expected to have grossly different chemical properties by virtue of the presence or absence of the hydrophobic N^6 side-group.

In order to provide a more critical and systematic approach to this problem, I synthesized and tested the biological activity of

a series of benzyladenine derivatives, each with a single halogen substitution in the N^6 benzyl side-group.

Although extensive studies have been made of the structure-activity relationship in single bioassays (e.g., Skoog's work with the tobacco callus bioassay (cf. Leonard, 1974) or Kuraishi's (1959) studies with the radish leaf expansion bioassay), there has been little or no effort to systematically compare the activity spectra of closely related cytokinin analogues in several bioassays. Although Letham (1967) compared the activities of zeatin, kinetin and 2iP in five bioassays, the small number of compounds tried and the considerable structural differences between them limit conclusions concerning the underlying reasons for differences in biological activities.

Materials and Methods

Synthesis of cytokinin analogues

ortho-F-, *meta*-F-, *para*-F-, *ortho*-Cl-, *meta*-Cl-, *para*-Cl-, *ortho*-Br-, and *para*-Br-Benzyladenine were synthesized from 6-methylmercaptopurine and the respective halogenated benzylamine by the procedure of Elion et al. (1952), as described by Okumura et al. (1959) and outlined in detail earlier for the synthesis of *p*-Br-BA (see Section I). In all cases, analytical samples were obtained by a single recrystallization from ethanol or dimethylformamide and purity and identity established by mass spectroscopic analysis (Figures 1 and 6-14) and Sephadex LH-20 chromatography (Figure 5, Table 2). The latter technique was used to detect the presence of non-derivatized BA. No BA was found in any sample, the minimum level of detection being approximately $\leq 0.5\%$.

Figures 6 through 14. Mass spectra of cytokinin analogues: *o*-F-BA, *m*-F-BA, *p*-F-BA, *o*-Cl-BA, *m*-Cl-BA, *p*-Cl-BA, *o*-Br-BA, N^6-methyl-*m*-Br-BA, and *m*-Br-BA. All spectra were obtained at 70 eV using a Varian Model CH-5 Mass Spectrometer.

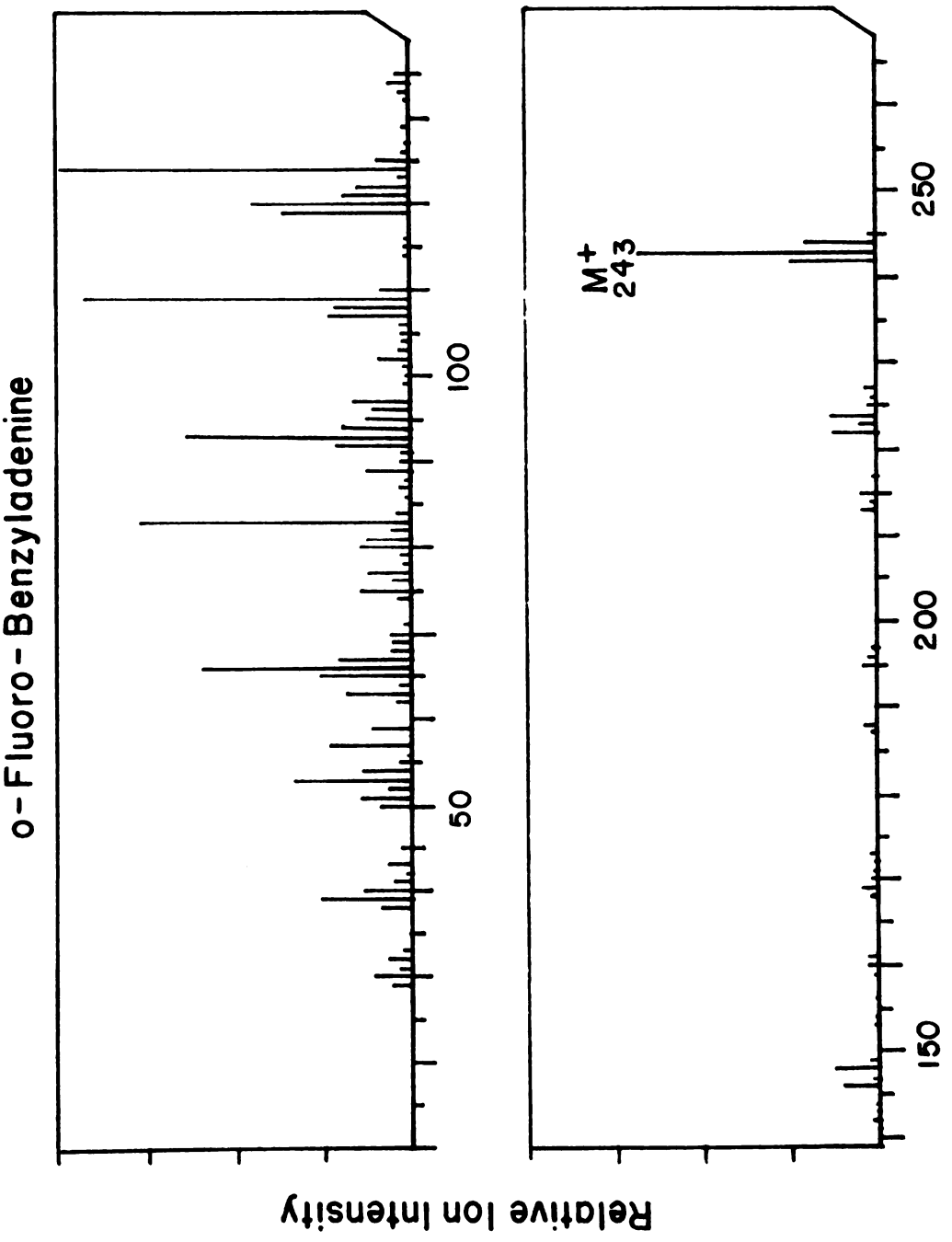

o-Fluoro-Benzyladenine
Relative Ion Intensity
50
100
150
200
250
m/e
M+
243

Figure 6

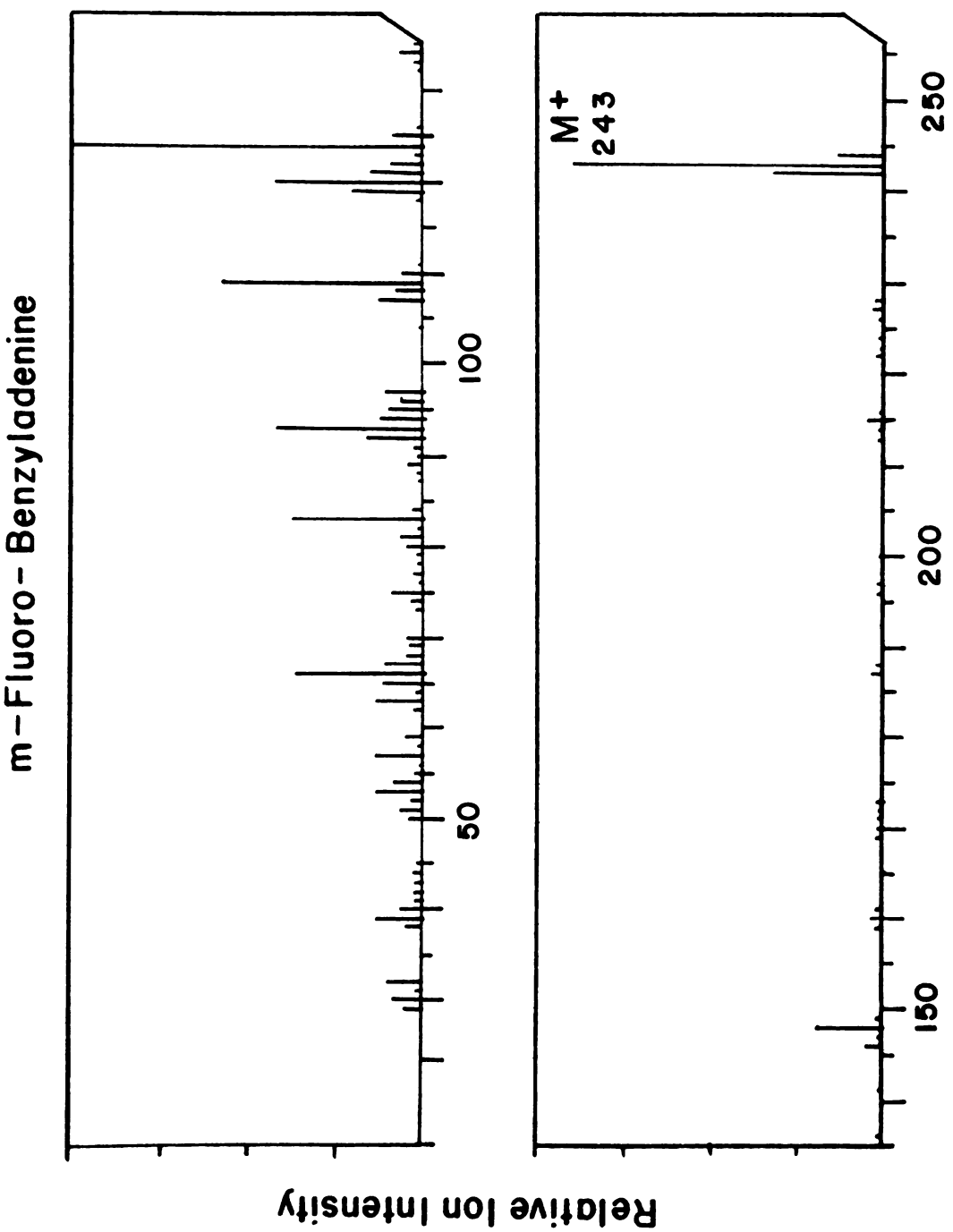

m-Fluoro-Benzyladenine
Relative Ion Intensity
50
100
150
200
250
m/e
M+
243

Figure 7

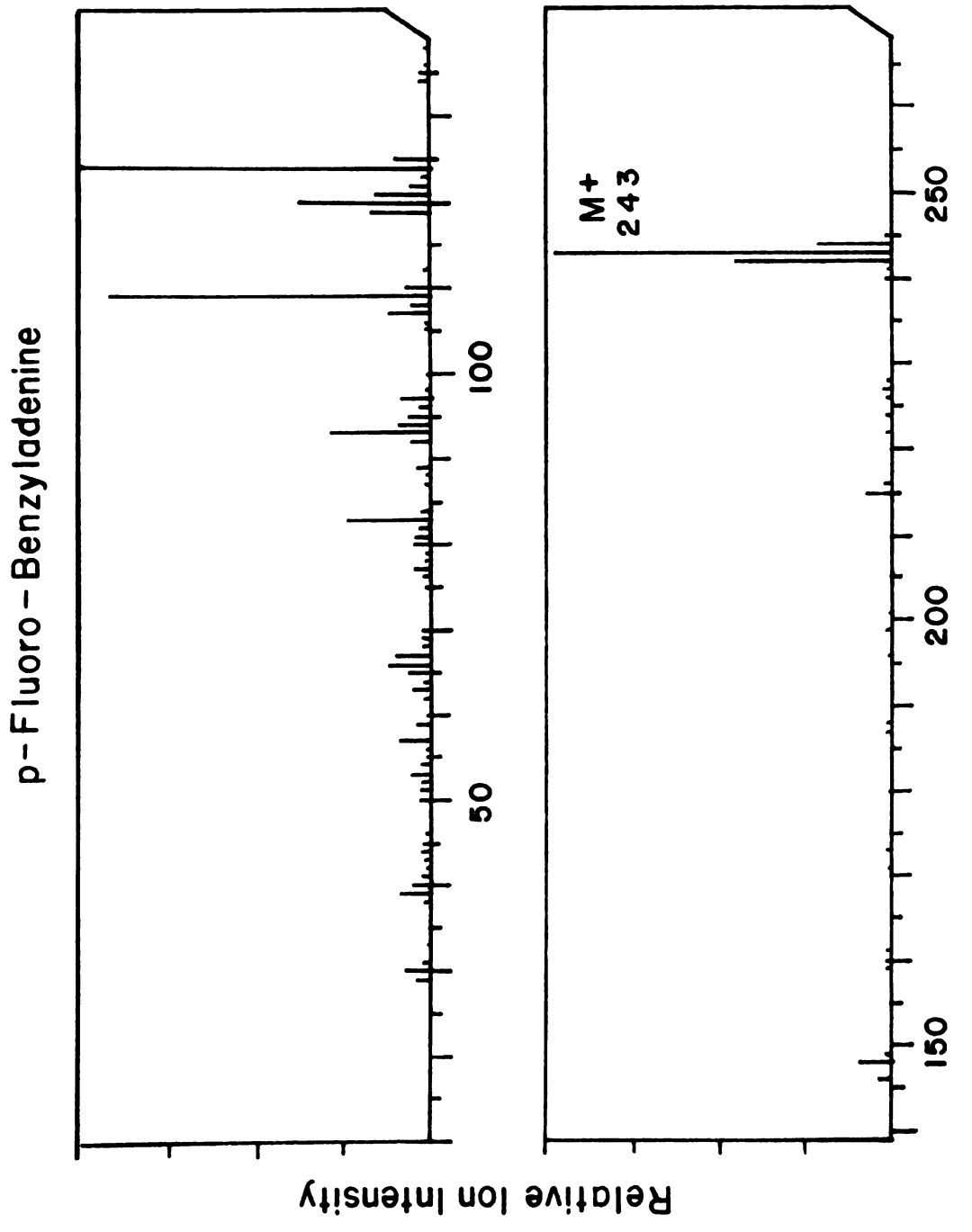

p-Fluoro-Benzyladenine
Relative Ion Intensity
50
100
150
200
250
m/e
M+
243

Figure 8

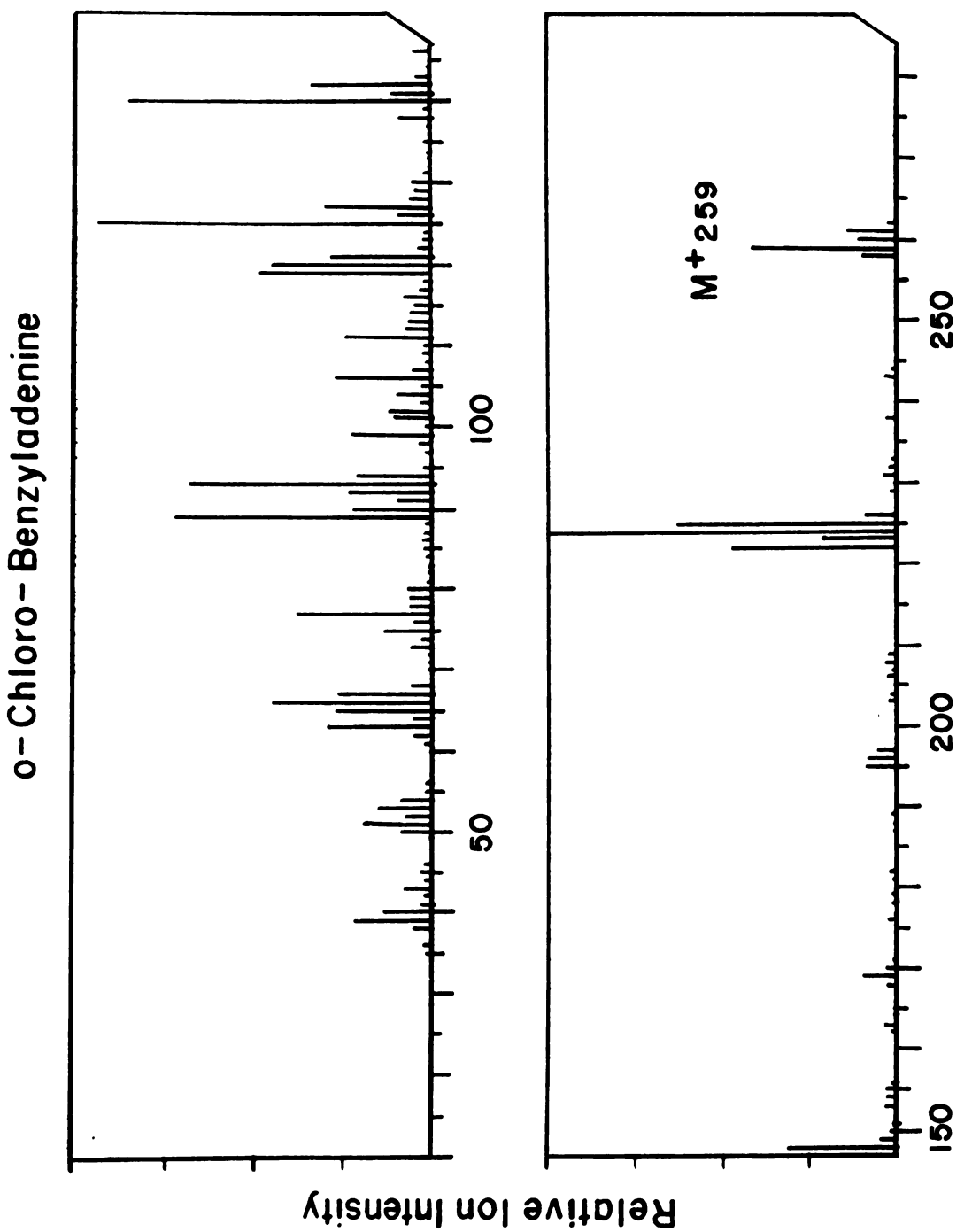

o-Chloro-Benzyladenine
Relative Ion Intensity
50
100
150
200
250
m/e
M+259

Figure 9

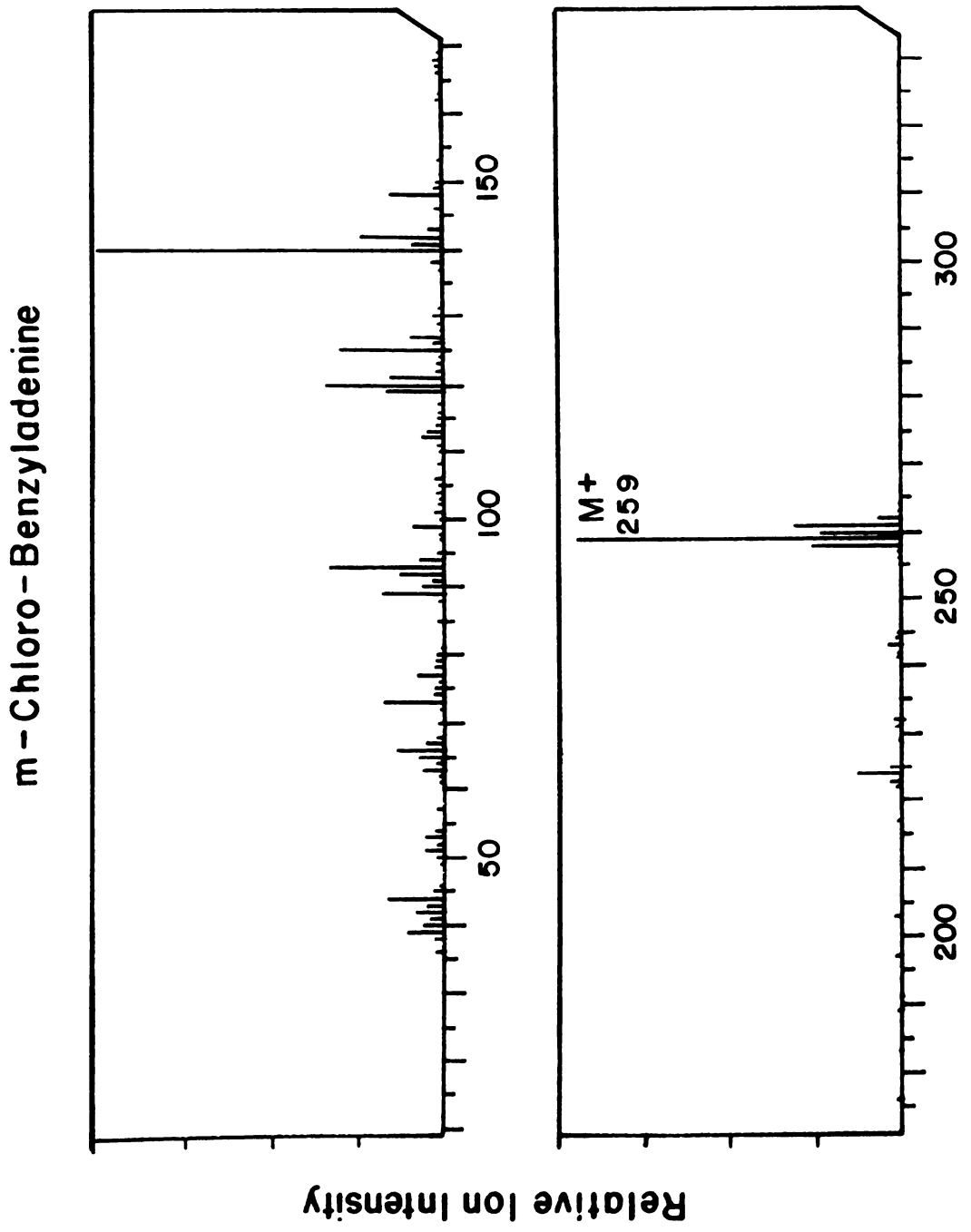

m-Chloro-Benzyladenine
Relative Ion Intensity
50
100
150
M+
259
200
250
300
m/e

Figure 10

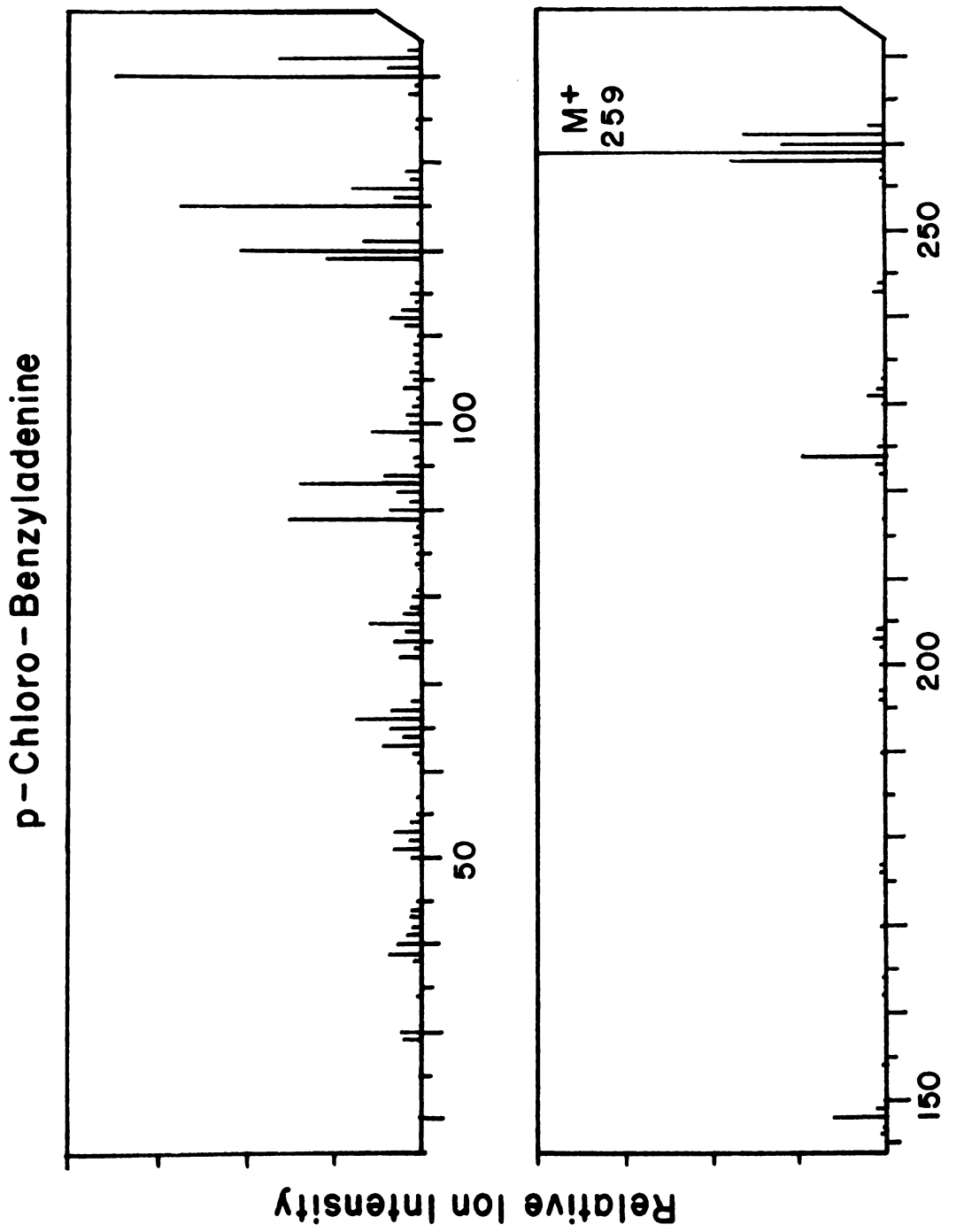

p-Chloro-Benzyladenine
Relative Ion Intensity
50
100
150
200
250
m/e
M+
259

Figure 11

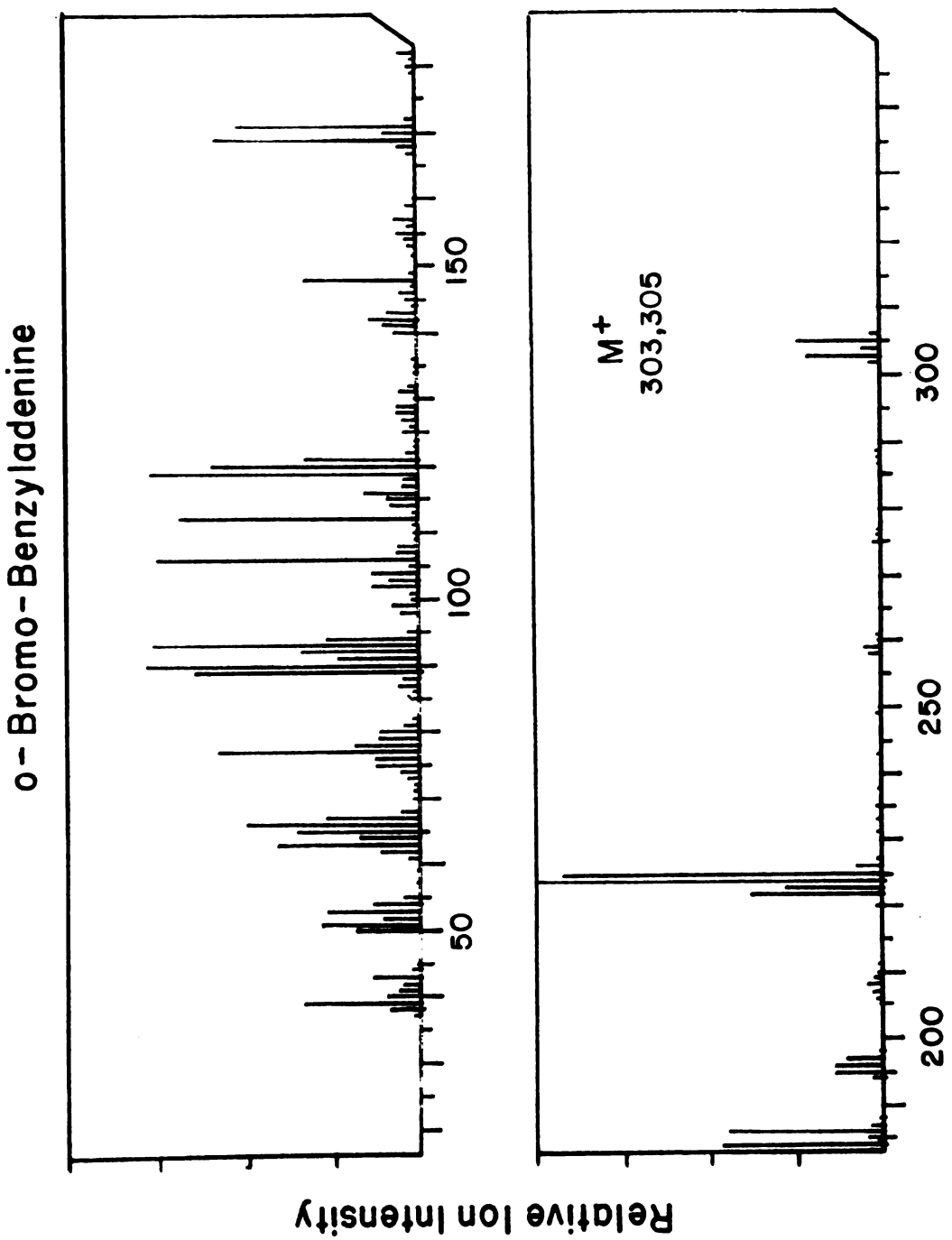

o-Bromo-Benzyladenine
M+
303,305
50
100
150
200
250
300
m/e
Relative Ion Intensity

Figure 12

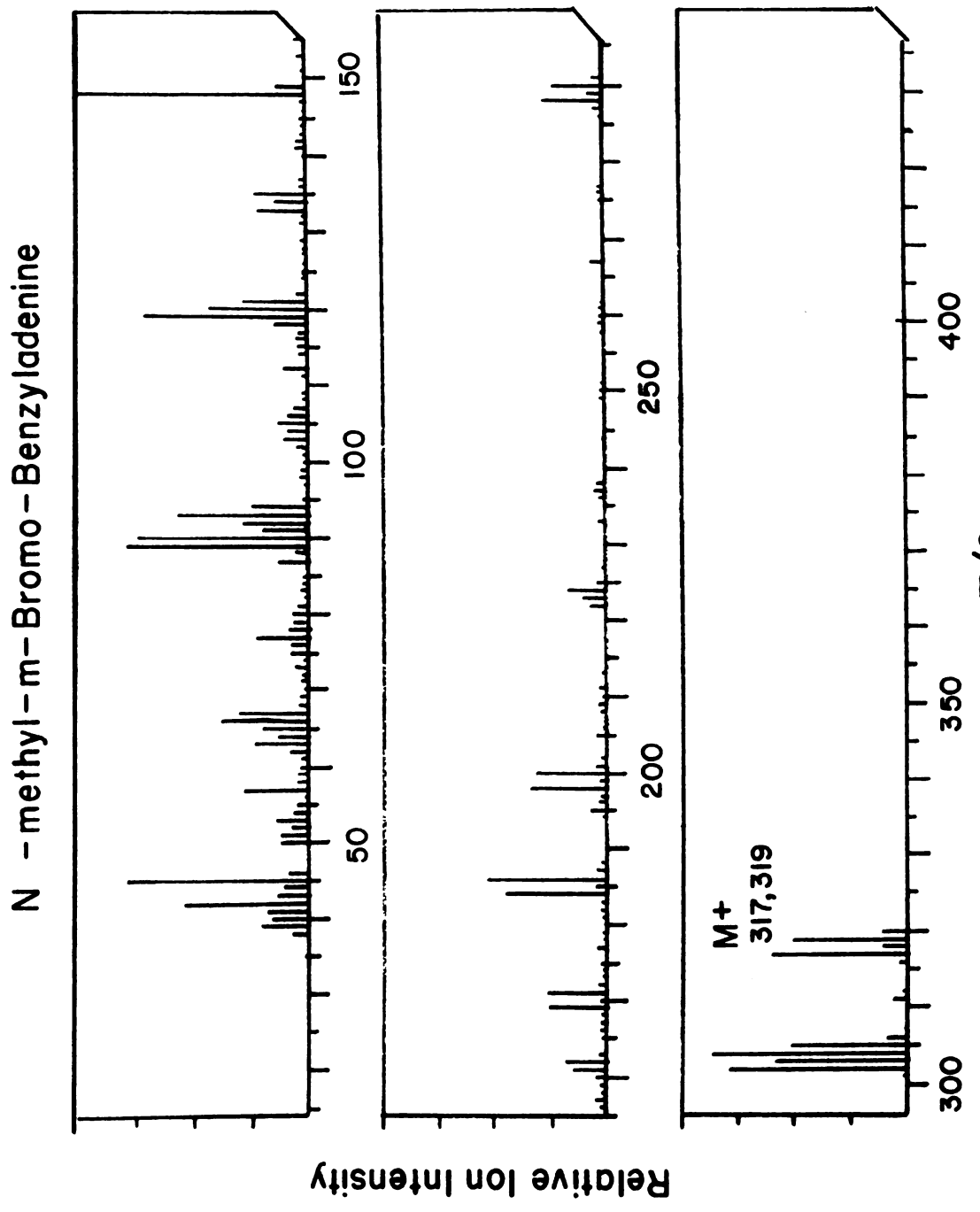

N -methyl-m-Bromo-Benzyladenine
Relative Ion Intensity
50
100
150
200
250
300
350
400
m/e
M+
317,319

Figure 13

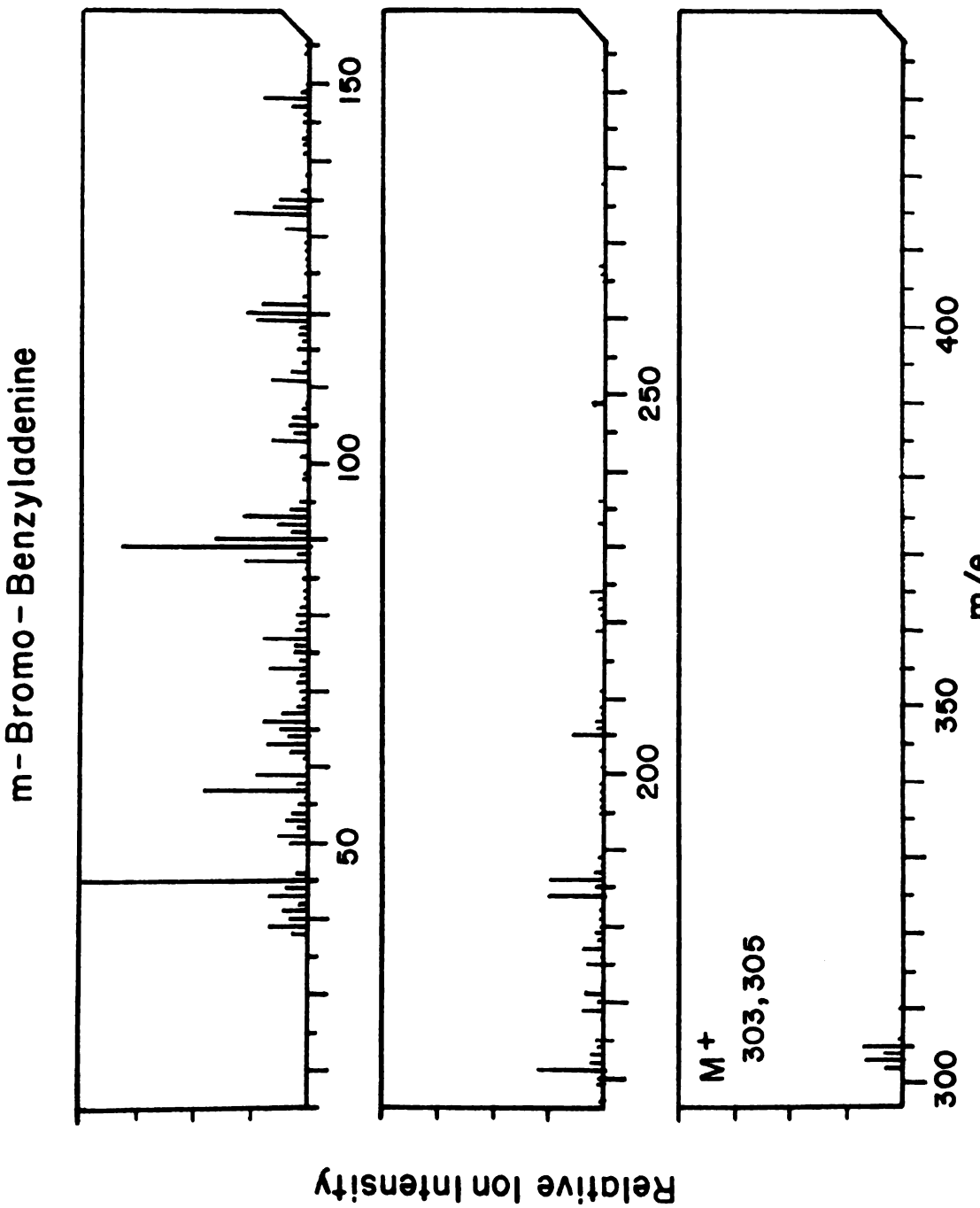

m-Bromo-Benzyladenine
Relative Ion Intensity
50
100
150
200
250
300
350
400
m/e
M+
303,305

Figure 14

Table 2. Sephadex LH-20 chromatographic mobility of cytokinin analogues

Compound[a]	Relative Mobility[b,c,d]
BA	2.9
o-F-BA	3.3
m-F-BA	3.5
p-F-BA	3.5
o-Cl-BA	4.2
m-Cl-BA	4.7
p-Cl-BA	4.9
o-Br-BA	5.0
p-Br-BA	5.6
BA-riboside	2.1
8-N_3-BA	4.1

[a] A list of the mobilities of additional cytokinin analogues is given by Armstrong et al. (1969).

[b] A relative mobility of 1.0 is given by an elution volume equal to one bed volume.

[c] The excluded volume is at a relative mobility of 0.3.

[d] Column dimensions and solvent as in Figure 5.

Mass spectroscopic analysis proved most valuable in showing that a sample of "*m*-Br-BA" which had appeared homogeneous on Sephadex LH-20 chromatography was in fact mostly another compound which sublimed in the mass spectrometer at a slightly lower temperature than *m*-Br-BA and which gave an unexpected molecular ion at *m/e* 317, 319. On the basis of its mass spectrum (Figure 13), this compound was identified as N^6-methyl-*m*-Br-BA. The formation of this N^6-methyl derivative was found to be caused by contamination of the commercially obtained *m*-Br-benzylamine with N-methyl-*m*-Br-benzylamine (K and K Rare and Fine Chemicals, Plainview, NY). Because of this, the "*m*-Br-BA" derivative was not further studied in the bioassays.

Tobacco cell suspension: growth characteristics and bioassay procedure

The cytokinin-dependent cell suspension used in this study was kindly supplied to us in June 1974 by Dr. C. Réaud-Lenoël (Centre Universitaire de Marseille-Luminy, Biochimie Fonctionnelle des Plantes, F-13288 Marseille, Cedex 2, France). It was strain #21 (Tandeau de Marsac and Jouanneau, 1972), an isolate originally obtained from tobacco pith (*Nicotiana tabacum* L.cv. Wisconsin 38). These cells were normally subcultured biweekly using a defined liquid medium (Jouanneau and Réaud-Lenoël, 1967) containing 2×10^{-7} M 2,4-D and 5×10^{-7} M kinetin. To obtain tissue routinely in sufficient quantity for *in vitro* binding studies (see Section III), 480 ml medium in 1-liter Erlenmeyer flasks was inoculated with approximately 2^5 ml of a 2-week-old culture. A growth curve under these conditions (Figure 15) showed a lag period followed by an exponential phase. By using larger inocula, the lag period could be shortened without a change in doubling time or final fresh weight yield.

Figure 15. Growth curve for cytokinin-dependent tobacco cells grown in liquid medium. Results are expressed as the mean fresh or dry weight ± S.E. of the tissue from triplicate flasks. Dry weight was measured after the cells were dried at 60°C for 2-3 days.

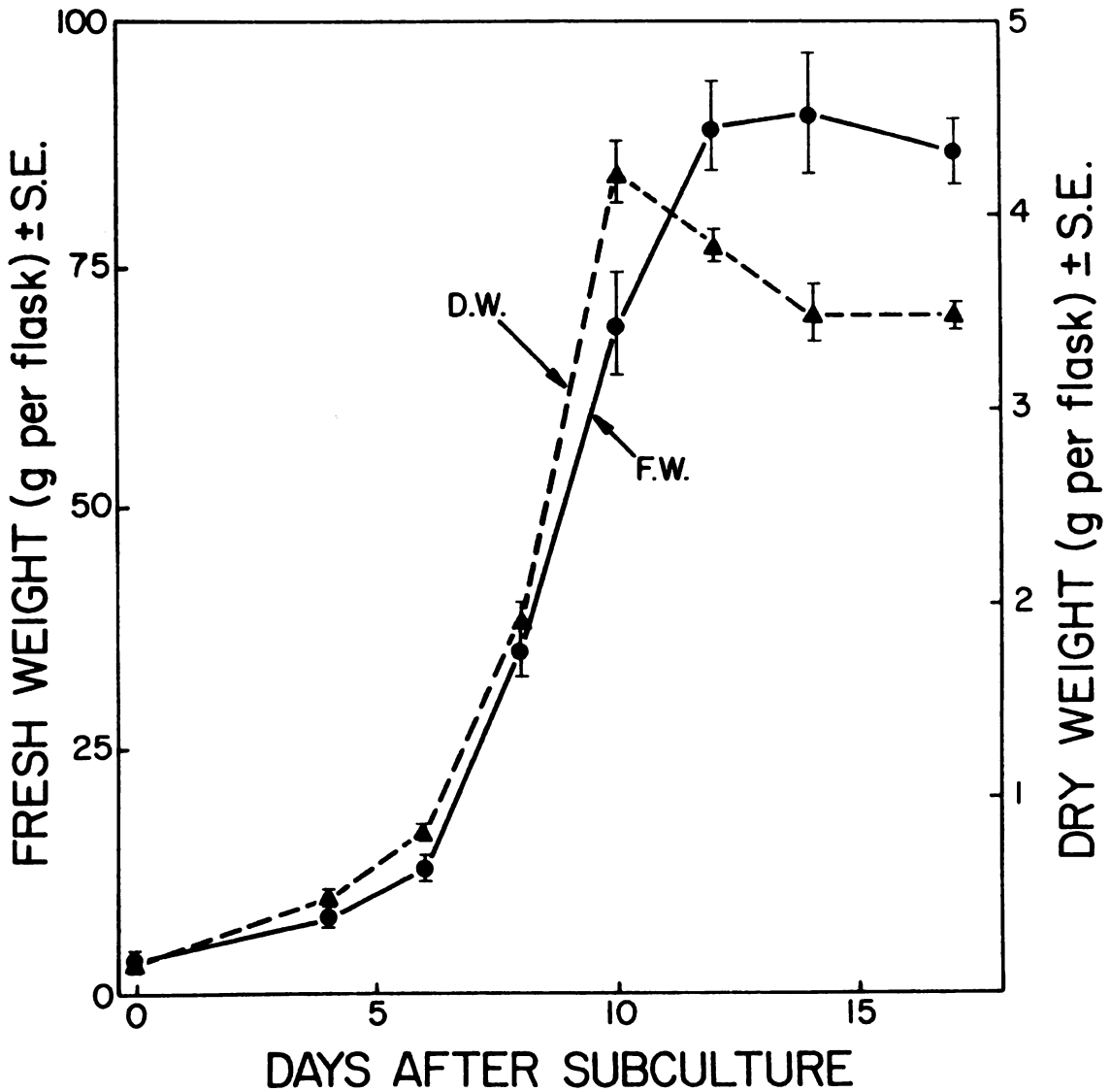

100
75
50
25
0
FRESH WEIGHT (g per flask) ± S.E.
5
4
3
2
1
DRY WEIGHT (g per flask) ± S.E.
D.W.
F.W.
0
5
10
15
DAYS AFTER SUBCULTURE

Figure 15

For bioassay, 250 ml Erlenmeyer flasks containing 60 ml medium were used. An inoculum free of the exogenously supplied kinetin was commonly obtained by aseptically collecting the cells from a 2-week-old culture on a Miracloth filter. The cells were then rinsed and resuspended in cytokinin-free medium and aliquots used as inocula for the bioassay. Culture flasks were agitated on a circular shaker at 125 rpm, under normal room lights, at 28°C $\pm$ 2°C. After 11 days (early stationary phase), the cells were collected by filtration on coarse fritted glass filters. Before weighing, the cells were rinsed with a solution of 40 mM sucrose, 2 mM Na_2-EDTA, 5 mM KCl, adjusted with NaOH to pH 5.7 at room temperature, in order to solubilize and remove accumulated extracellular polysaccharides (A. Hanson, personal communication).

Moss bioassay

Cytokinin-induced bud formation in protonemata of the moss, *Funaria hygrometrica* (L.) Sibth. was measured as described by Whitaker and Kende (1974). Buds were counted 48 hr after transfer of protonemata to cytokinin-containing medium.

Agrostemma bioassay

Enhancement of nitrate reductase activity by cytokinins in excised embryos of *Agrostemma githago* was measured 5 hr after start of the hormone treatment. The procedure of Kende et al. (1971) was followed.

Tobacco callus differentiation bioassay

Induction of shoot formation in tobacco callus was examined using callus which had been grown from pith (*Nicotiana tabacum* L.cv.

Wisconsin 38) isolated no longer than 9 months previously. This callus was subcultured every 6-7 weeks on standard Linsmaier-Skoog medium (Linsmaier and Skoog, 1965) with indoleacetic acid (10^{-5} M) and kinetin (10^{-7} M). To determine the optimal auxin and cytokinin concentrations for the shoot formation assay, explants were placed on the same minimal medium but containing various concentrations of BA and 2,4-D. The results (Figure 16) showed that callus formation alone was maximal on medium with 5×10^{-7} to 1×10^{-6} M BA and 10^{-6} M 2,4-D but shoot formation was maximal on medium containing 5×10^{-6} to 1×10^{-5} M BA in the absence of 2,4-D.

Thus, for the differentiation assay with cytokinin analogues, 3 callus explants (approximately 0.4 g each) were placed in each of five 125-ml Erlenmeyer flasks containing 50 ml medium with various concentrations of the test substance, but no auxin. Tissue was grown for 6 weeks in the dark, followed by 1 week under room lights. I attempted to quantitate shoot formation (Table 3) by two methods: (1) physically separating undifferentiated callus tissue from shoot and leaf tissue and weighing each separately and (2) measuring the chlorophyll *concentration* in the entire explant by extracting with a three-fold (ml/g F.W.) volume of 95% ethanol. The second method was feasible because only the shoots and leaves greened up appreciably. The two methods gave qualitatively similar results concerning the relative amounts of shoots and undifferentiated callus within each treatment.

Figure 16. Determination of the optimum auxin (2,4-D) and cytokinin (BA) concentrations for callus and for shoot formation in tobacco callus grown on solid medium. Results are expressed as the mean fresh weight of the tissue from quintruplicate flasks, after a culture period of 3-1/2 weeks.

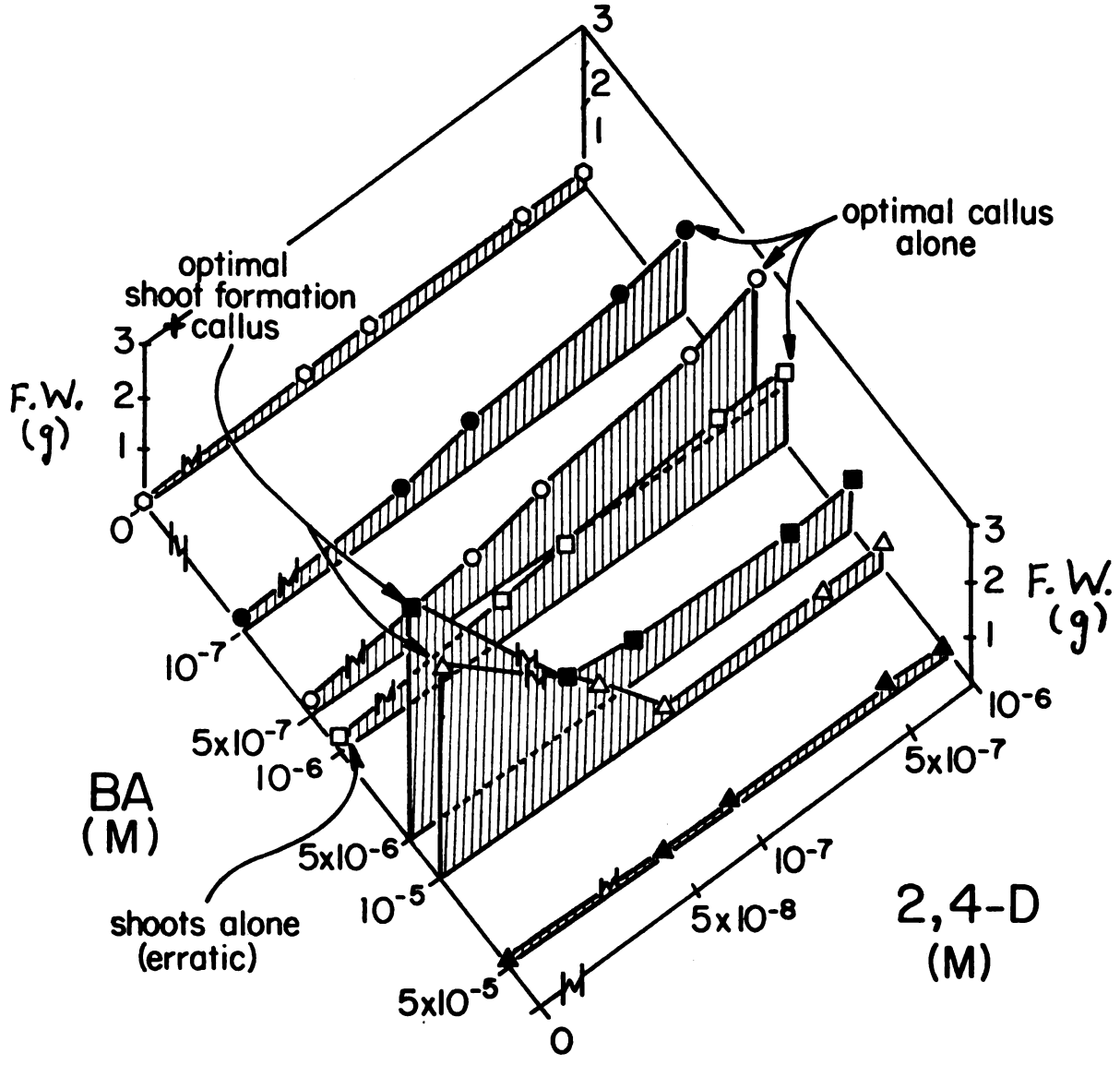

3
2
1
optimal callus alone
optimal shoot formation callus
F.W. (g)
3
2
1
0
M
10-7
5x10-7
10-6
5x10-6
10-5
5x10-5
0
BA (M)
shoots alone (erratic)
3
2
1
F.W. (g)
10-6
5x10-7
10-7
5x10-8
2,4-D (M)

Figure 16

Results

Physical and chemical properties of *ortho-*, *meta-*, and *para*-halogenated BA derivatives

The observed elution order of the cytokinin analogues in adsorption chromatography on an alkylated Sephadex support, Sephadex LH-20, in 35% ethanol was (Table 2), BA > *o*-F-BA > *m*-F-BA > *p*-F-BA > *o*-Cl-BA > *o*-Cl-BA > *m*-Cl-BA > *p*-Cl-BA > *o*-Br-BA > *p*-Br-BA. This parallels their expected order of lipophilicity (i.e., *para* > *meta* > *ortho*, according to Kuraishi, 1959). There is apparently little separation of these BA derivatives on the basis of molecular size since this would cause an elution order of Br < Cl < F, which is exactly reverse of the observed one.

Although the overall fragmentation pattern of the derivatives (Figures 1 and 6-14) was very similar to that obtained with BA (Figure 1), there was a systematic difference in the intensities of certain ions relative to each other. The most notable difference was an increase in the relative intensity of the following ions in the spectra of the *ortho*-substituted derivatives (X = halogen): (1) $M^+ - X$; (2) $C_7H_5X^+$, the substituted tropylium ion; (3) $C_6H_4X^+$; and (4) $M^+ - X + 1$. The increased intensitives of the first three ions can possibly be regarded as being due to an increase in lability of the respective bonds because of a 6-membered ring formed through hydrogen bonding of the *ortho*-substituted halogen to the N^6-amino hydrogen (Figure 21).

Biological properties of *ortho-*, *meta-* and *para*-halogenated BA derivatives

In the tobacco cell suspension bioassay the relative activities of the halogenated BA derivatives were (Figure 17): *o*-F-BA = *m*-F-BA = *p*-F-BA = BA > *o*-Cl-BA $\geq$ *o*-Br-BA > *m*-Cl-BA $\geq$ *p*-Cl-BA = *p*-Br-BA (inactive). In the moss bioassay, the order of activity was (Figure 18): *m*-Cl-BA $\geq$ *o*-F-BA = *m*-F-BA = *p*-F-BA = BA > *p*-Cl-BA $\geq$ *o*-Cl-BA = *o*-Br-BA > *p*-Br-BA (inactive). In the *Agrostemma* bioassay, a limited number of analogues was tested, and their observed order of activity was (Figure 19): BA = *m*-Cl-BA > *o*-Cl-BA > *p*-Cl-BA. The other derivatives were less extensively tested in this bioassay, but in general, they were found to follow the same order as in the moss and tobacco assays (i.e., *o*- and *p*-F-derivatives completely active and *p*-Br-BA completely inactive).

In an experiment (Table 3) designed to measure the activities of cytokinin analogues in inducing differentiation of shoots in tobacco callus, the results showed that BA and *o*-Cl-BA were both slightly more active than *m*-Cl-BA. However, a striking feature of this experiment was the ability of *m*-Cl-BA (at 5×10^{-6} M and 10^{-5} M) to cause shoot formation in almost the complete absence of callus formation. With this compound, the shoots appeared to arise from a brown, necrotic piece of callus (Figure 20). This result was consistently obtained in each of the three repeats of this experiment. A similar, but more erratic, response was obtained at the lower (10^{-6} M) concentrations of BA and *o*-Cl-BA (Table 3).

Discussion

A result which was common to all three bioassays was that in any one position of the benzene ring, the biological activity of the

Figure 17. Cytokinin activity of halogenated-BA derivatives in a tobacco suspension culture cell division bioassay. Results are expressed as the mean ± S.E. of the tissue from at least quadruplicate flasks.

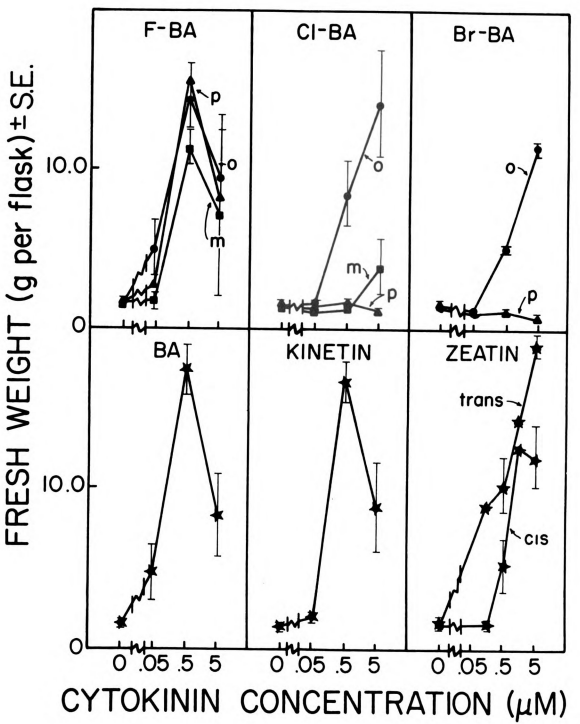

F-BA
Cl-BA
Br-BA
BA
KINETIN
ZEATIN
p
o
m
trans
cis
10.0
0
0 .05 .5 5
FRESH WEIGHT (g per flask)± S.E.
CYTOKININ CONCENTRATION (μM)

Figure 17

Figure 18. Cytokinin activity of halogenated BA derivatives in the moss bud formation bioassay. Results are expressed as the mean ± S.E.of three replicate samples.

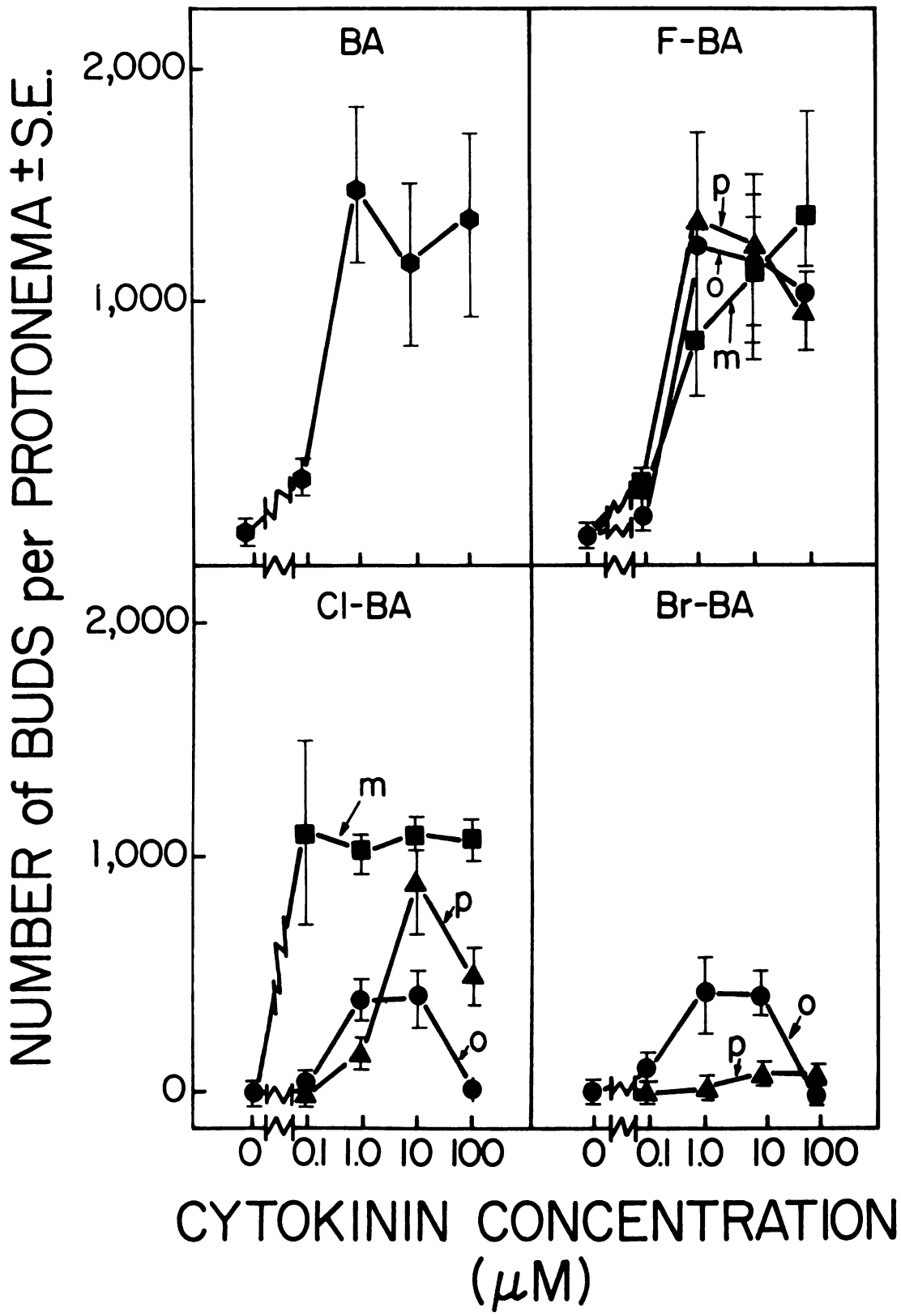

BA
F-BA
Cl-BA
Br-BA
2,000
1,000
0
p
o
m
0 0.1 1.0 10 100
NUMBER of BUDS per PROTONEMA ± S.E.
CYTOKININ CONCENTRATION
(μM)

Figure 18

Figure 19. Cytokinin activity of halogenated-BA derivatives in the bioassay measuring the induction of nitrate reductase in excised *Agrostemma githago* embryos. Results are expressed as the mean $\pm$ S.E. of three replicate samples.

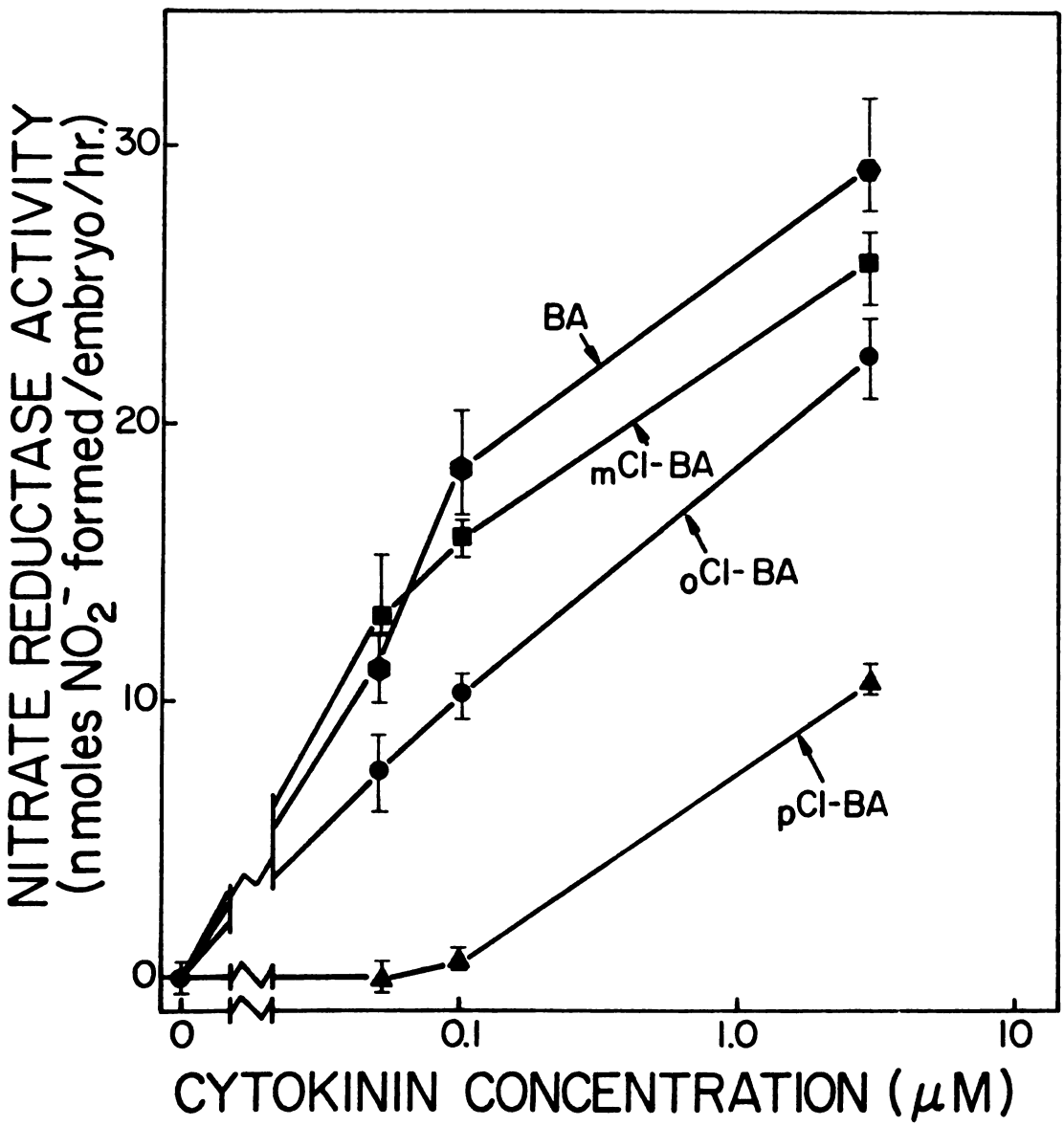

NITRATE REDUCTASE ACTIVITY
(n moles NO_2^- formed/embryo/hr.)
30
20
10
0
BA
mCl-BA
oCl-BA
pCl-BA
0
0.1
1.0
10
CYTOKININ CONCENTRATION (μM)

Figure 19

Table 3. Effect of cytokinin analogues on shoot formation in tobacco callus

Compound	Fresh Weight per Flask (g)[a]			Shoots/Callus (g/g)	A_{652} of Extract[b,c]
	Shoots	Callus	Total		
H_2O	0.0	0.4 ± 0.1	0.4 ± 0.1	-	-
BA, 10^{-6} M	12.3 ± 1.5	1.3 ± 0.6	13.6 ± 1.6	9.6 ± 2.1	0.635 ± .002
BA, 5 x 10^{-6} M	24.8 ± 1.2	3.9 ± 0.4	28.7 ± 1.2	6.4 ± 1.6	0.378 ± .032
BA, 10^{-5} M	13.8 ± 2.8	4.5 ± 0.8	18.3 ± 2.9	3.1 ± 3.6	0.284 ± .027
o-Cl-BA, 10^{-6} M	12.5 ± 4.3	0.7 ± 0.2	13.2 ± 4.3	18.7 ± 4.5	0.791 ± .107
o-Cl-BA, 5 x 10^{-6} M	21.4 ± 3.7	3.1 ± 0.8	24.5 ± 3.8	6.8 ± 4.5	0.366 ± .030
o-Cl-BA, 10^{-5} M	25.8 ± 1.6	5.2 ± 0.6	31.0 ± 1.7	5.0 ± 2.2	0.247 ± .012
m-Cl-BA, 10^{-6} M	0.0	0.7 ± 0.2	0.7 ± 0.2	-	-
m-Cl-BA, 5 x 10^{-6} M	15.5 ± 2.8	0.9 ± 0.2	16.5 ± 3.0	16.5 ± 3.0	0.765 ± .098
m-Cl-BA, 10^{-5} M	15.7 ± 3.7	0.9 ± 0.2	16.6 ± 3.7	17.8 ± 3.9	0.614 ± .052
p-Cl-BA, 10^{-5} M	0.0	0.4 ± 0.1	0.4 ± 0.1	-	-

[a]Three callus explants (0.4 g each) were cultured in each of five 125 ml Erlenmeyer flasks containing 50 ml basal medium lacking auxin. Results are expressed as the mean fresh weight of tissue per flask ± S.E. after a 7 week culture period (see Materials and Methods).

[b]Chlorophyll concentration *per flask* was estimated by measuring the A_{652} of an alcoholic extract of the tissue (95% ethanol, 3 ml/g F.W.).

[c]Callus alone (no shoots) gave an A_{652nm} of 0.190 ± .023 at 1 g of tissue per 3 ml of 95% ethanol.

Figure 20. Pictorial comparison of the effects of BA, *o*-Cl-BA, *m*-Cl-BA and *p*-Cl-BA on shoot formation in solid-grown tobacco callus. The results shown are representative contents of one flask after growth for 7 weeks on medium containing the test compound at 10^{-5} concentration.

Figure 20

halogenated BA derivatives followed this order: F = H > Cl > Br. This relationship correlates with the order of their atomic size (H > F > Cl > Br) but not their electronegativity (F > Cl > Br > H).

The major difference which was observed between the bioassays concerned the relative activities of the *o*- and *m*-Cl-BA derivatives. Thus, in the tobacco cell division assay, *o*-Cl-BA is active (but less so than BA) and *m*-Cl-BA is almost inactive. In the moss bud formation assay, however, the reverse is true; i.e., *m*-Cl-BA is fully active and *o*-Cl-BA is much less active. In the *Agrostemma* assay, *m*-Cl-BA is as active as BA, as in the moss bud formation assay; however, *o*-Cl-BA is also active, though significantly less active than BA, exactly as in the tobacco cell division assay. Thus, apparently the specificity of the *Agrostemma* response can be construed as a *summation* of the activities observed in the tobacco cell division and moss bud formation assays.

In the discussion to follow, I assumed as the simplest and most likely situation that the structural requirements for biological activity observed in these bioassays is solely a reflection of differences in the respective cytokinin binding requirements at a receptor site, and that metabolism and uptake do not contribute to the differences in biological activities of these analogues. There are no data in any plant system to confirm or refute this assumption. In light of this, I have clearly labelled each of the following headings and conclusions as "speculative." Regardless of whether or not a receptor is the only protein involved, the reader should be aware that the differences in biological activities of cytokinins in different bioassays are most likely due to differences in a specific cytokinin binding site(s) of a protein(s) since it is highly unlikely that a

purely physical process, such as uptake through membrane diffusion, could be different in moss, as compared to tobacco.

Speculations on the distribution in nature of cytokinin receptors and the topography of their cytokinin binding sites

(i) Tobacco cell division receptor. Kuraishi (1959), using BA and kinetin analogues with substituted N^6 side-groups, and the radish leaf expansion bioassay, interpreted differences in biological activity of cytokinin analogues on the basis of the lipophilicity of the N^6 side-group. However, in order to explain a decrease in biological activity when the lipophilic properties were increased (such as when there were 6 or more C atoms in a linear saturated hydrocarbon N^6 side-group), he postulated that too high a lipophilic value is as bad as too low a value. However, using the R_f on Sephadex LH-20 (Table 2) as a relative measure of lipophilicity, Kuraishi's hypothesis does not explain why *o*-Br-BA is more active than *p*-Cl-BA in the tobacco cell division assay (Figure 17).

An alternative explanation of the results of the tobacco cell division bioassay is summarized in a "steric" model illustrated in Figure 21. According to this hypothesis, reduced activity with the bulky *meta*- and *para*-derivatives is caused by the inability of these derivatives to bind to a receptor, because the N^6 side-group does not fit into a hydrophobic cleft of the protein. *Ortho*-substitutions are tolerated because substitution at this position would not be required to penetrate the cleft. The *ortho*-halogenated BA derivatives may also have a more favored conformation for biological activity because of hydrogen bonding between the halogen atom and the N^6-amino hydrogen. Soriano-Garcia and Parthasarthy (1975) reported that the crystal

Figure 21. A "steric" model of the cytokinin binding site of a cytokinin receptor in tobacco cells. This model explains the results observed for the structure-activity relationships with halogenated-BA derivatives in a tobacco cell division bioassay. For simplicity, only the N^6 side-group binding site is shown, though obviously the purine moiety is also bound since it too is required for activity (cf. Hecht et al., 1975).

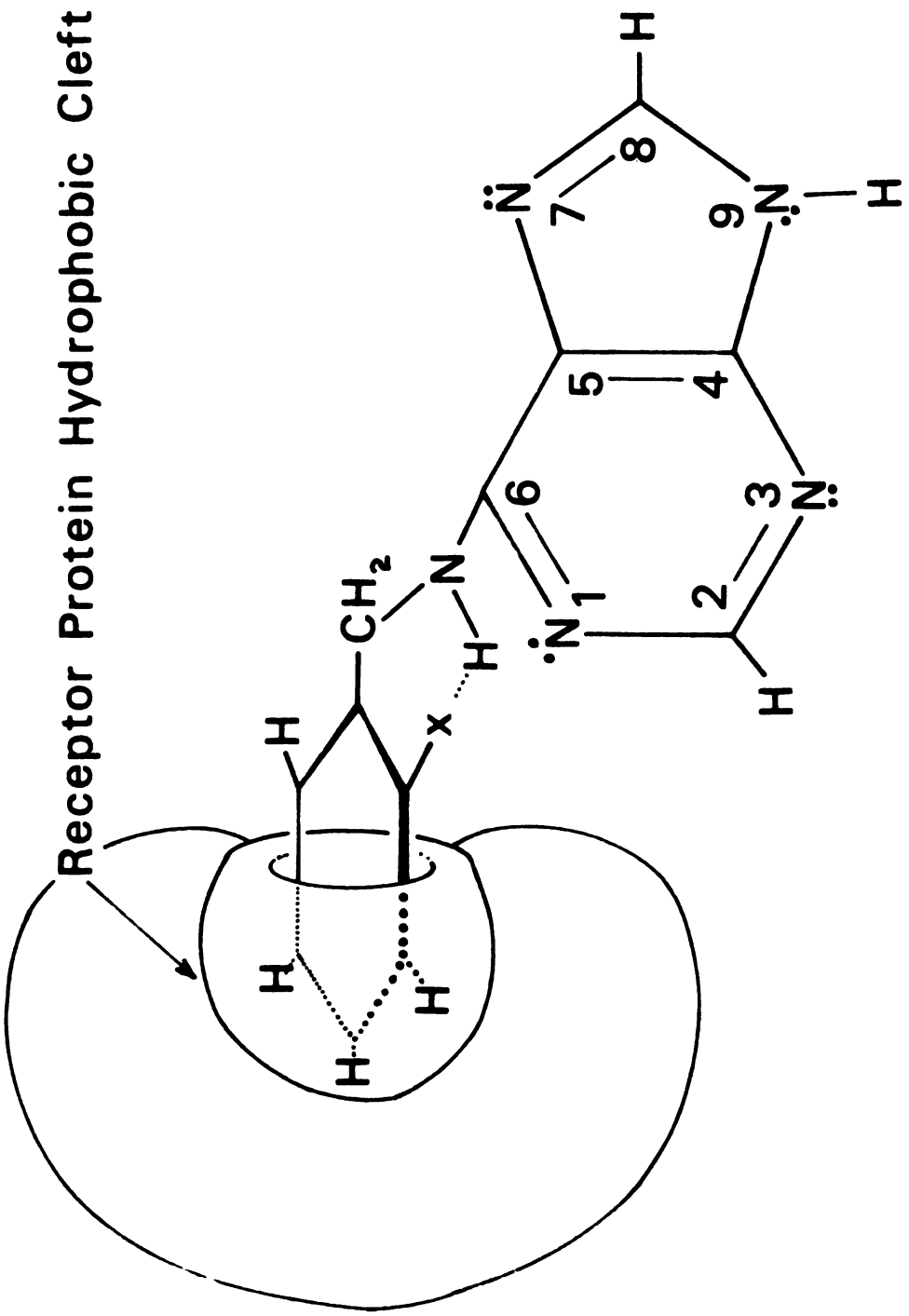

Receptor Protein Hydrophobic Cleft
H
N
8
7
9
N
H
5
4
6
3
N
N
1
2
H
H
CH2
N
H
H
X
H
H
H

Figure 21

structure of cytokinins such as BA is best described as: (1) two planes at approximately right angles to each other, one plane being defined as passing through the 10 atoms of the adenine moiety and the other plane passing through the benzyl group; (2) the N^6-benzyl group is distal to the imidazole ring. Thus, the internal hydrogen bonding might allow a more favored conformation for biological activity by "cementing" the benzene ring into a plane more perpendicular to the purine moiety. A similar interpretation was also proposed by Dyson et al. (1972) to explain the 1,000-fold increase in activity of *o*-chlorophenylureidopurine over phenylureidopurine, observed in a soybean callus assay.

(ii) Cytokinin receptor in moss protonemata. The high activity of *m*-Cl-BA coupled with the low activity of both *o*- and *p*-Cl-BA indicates that binding to the receptor in moss protonemata involves the electron resonance properties of the N^6 benzyl side-group. The ability to accommodate a bulky chlorine group in the *meta*-position demonstrates the absence of steric restraints on binding, in contrast to the model for tobacco cells. Advantage can be taken of this observation in the design of active-site directed irreversible reagents for the moss receptor; likely candidates are the *meta*-substituted mercapto-, mercuro-, diazo-, or azido-BA derivatives.

(iii) Are there separate cytokinin receptors for stimulating cell division and differentiation (shoot formation) in tobacco cells? As discussed above, the specificity observed in the *Agrostemma* assay could be explained by assuming that both a moss bud formation-like receptor and a tobacco cell division-like receptor were operating simultaneously in this tissue.

In the same context, I hypothesized that the tobacco cell division "receptor" and moss bud formation "receptor" were mediators of two fundamental, independent cytokinin reactions, one leading to a stimulation of cell division (tobacco cell division response) and one leading to differentiation (moss bud formation response). An attempt to test this hypothesis was made by comparing the differentiation-stimulating activity (shoot formation) of cytokinin analogues with their cell division-stimulating activity in the *same* tissue. At face value, the results indicate that the order of activities of the analogues is the same for both responses. However, the ability of *m*-Cl-BA to more consistently induce shoot formation in the complete absence of callus formation at all concentrations also suggests that the original hypothesis may be correct, but that the two responses were not adequately separated in the tobacco shoot formation assay. Such a difficulty is not unexpected since the process of shoot formation (differentiation) will clearly require cell division and, therefore, a compound such as *o*-Cl-BA could have its differentiation-stimulating activity overestimated, by virtue of its strong cell division stimulating activity.

This hypothesis is testable in a more definitive experiment which could be performed if a compound were found which was active in the tobacco cell division assay but inactive in the tobacco shoot formation assay. The activity of cytokinin analogues in stimulating tobacco shoot formation could then be measured independently of cell division simply by testing cytokinin derivatives in the shoot formation assay in the presence of this cell division-active, shoot formation-inactive compound. Thus, by pre-saturating the cell division response with this compound, the only additional effect

which would be noticed is the stimulation of shoot formation due to the differentiation response. If the two-receptor scheme I proposed is correct, potential candidates for this compound are metabolically stable, N^9-substituted derivatives since such compounds are active in tobacco and soybean cell division assays (Fox et al., 1973) but markedly inactive in the moss bud formation assay (Whitaker and Kende, 1974).

SECTION III

IDENTIFICATION OF A SPECIFIC CYTOKININ BINDING SITE IN A PARTICULATE FRACTION FROM TOBACCO CELLS

Introduction

As discussed earlier, one approach to the study of the mechanism of cytokinin action, which has not yet been successfully pursued, is the identification of the receptor(s) which binds the hormone. The results of Kende (Brandes and Kende, 1968), Skoog and co-workers (Hecht et al., 1975) suggest that this binding is non-covalent and should be amenable to identification *in vitro*. I thus undertook studies using radioactive benzyladenine to identify and localize *in vitro* the specific binding site(s) which may act as the cytokinin receptor(s).

Because of current interest in membranes as sensory transducers and the availability of a suitable binding assay, we have focused our attention on membranous cell fractions. In addition, preliminary experiments with a soluble cytosol fraction using equilibrium dialysis and Sephadex G-25 chromatography were unsuccessful in detecting significant amounts of specific binding.

The centrifugation binding assay as a technique for detecting particulate binding sites for plant hormones was originally pointed out by Hertel et al. (1972) in their *in vitro* auxin and 1-N-naphthylphthalamic acid binding studies as the technique of choice because it posesses the unique characteristic of great sensitivity and low background noise. It is also most useful for measuring under equilibrium

conditions, binding which is easily dissociated and in which the concentration of binding sites is very low. Thus, Hertel et al. (1972) were able to detect and quantitate specific binding which only represented 0.1-0.5% of the total radioactivity (i.e., bound/free ligand = 0.001-0.005).

Because of anticipated problems caused by large amounts of non-specific binding (Bezemer-Sybrandy and Veldstra, 1971), we have paid special attention to the structural specificity required for binding, as it compares to the structural specificity of the biological response in the same tissue. This has been made possible by the use of the same cytokinin-dependent tobacco suspension culture for both binding assays and bioassays (see Section II).

In my initial binding studies in 1972 I used ^{14}C-BA of low specific radioactivity since this was the most readily available radioactive cytokinin at that time. The preliminary results looked promising, showing the existence of pronase-sensitive binding of ^{14}C-BA which was saturable ($K_d \sim 10^{-6}$ to 10^{-5} M) and which was specific for biologically active hormones (i.e., kinetin, BA and 2iP competed for binding, while adenine and the cytokinin ribosides did not).

However, it was observed that (1) this binding *increased* almost two-fold after a heat pre-treatment (100°C, 15 min), (2) this binding could not be enriched in any fraction obtained by differential centrifugation, (3) treatments which increased non-saturable binding (pre-heating, KCl) also increased saturable binding and treatments which lowered non-saturable binding (pronase, Triton X-100) also lowered saturable binding, and (4) biologically inactive analogues (e.g., *p*-Cl-BA and *p*-Br-BA) which were chemically similar to active

cytokinins were active in the binding assay. I therefore concluded that it was probably *not* the result of cytokinin binding to a specific cytokinin receptor protein, but instead was a non-specific and physiologically irrelevant retention of BA.

Since it has been observed with animal hormones and with drugs that the physiologically important *high*-affinity binding of the ligand to a small number of receptor proteins may be undetectable because of the low specific activity of the radioactive ligand (Cuatrecasas et al., 1974) and because of an excess of lower-affinity, non-specific binding, I decided to test whether a similar situation existed with cytokinin binding in tobacco cells. Thus, a tritiated cytokinin, *p*-[^{3}H]-BA, of high specific activity (500-fold higher than that of the ^{14}C-BA previously used) was synthesized (Section I). The following is a report of the results which were obtained using this *p*-[^{3}H]-BA to detect specific *high*-affinity cytokinin binding sites in cell-free particulate fractions of tobacco cells.

Materials and Methods

Tobacco cell culture

Cytokinin-dependent tobacco cells were grown in suspension culture and harvested at mid- to late exponential phase (9-10 days of culture), as described earlier (Section II). Control experiments verified that rinsing the cells to remove extracellular polysaccharides prior to homogenization did not affect their subsequent growth characteristics or cytokinin requirements.

Preparation of cell-free particulate fractions

Cells were broken by homogenization in grinding medium (2 ml medium per g F.W. tissue) with a Super Dispax Tissumizer Model SD45 (Tekmar Co., P.O. Box 37202, Cincinnati, OH 45222) using a G454 generator at approximately 8,000 rpm for 30-45 sec. At this and all subsequent steps, solutions were maintained at 0-4°C. Intact cells were removed by filtration through 2 layers of Miracloth. An 80,000 x *g* particulate fraction was prepared by centrifuging the Miracloth filtrate at 30,000 rpm in a Beckman Preparative Ultracentrifuge (Type 30 rotor) or at 32,000 rpm in an International Preparative Ultracentrifuge (Type A-147 rotor). The pellets were resuspended in binding assay medium by two up and down strokes with a glass homogenizer and mechanically driven, tight-fitting Teflon pestle.

For the differential centrifugation experiment (Table 8), the 3 particulate fractions were obtained by centrifugation of each lower-speed supernatant at 13,000 x *g* (Sorvall Centrifuge, SS-34 rotor, 10,500 rpm), 80,000 x *g* (as above) and 170,000 x *g* (Beckman Preparative Ultracentrifuge, Ti50 rotor, 50,000 rpm, and International Preparative Ultracentrifuge, A-321 rotor, 55,000 rpm). The pellets obtained after each centrifugation were resuspended in binding assay medium as above.

Cytokinin-binding assay

Particulate fractions suspended in binding assay medium were incubated with radioactive BA plus or minus non-radioactive BA or cytokinin analogue for 1 hr at 0-4°C to reach equilibrium prior to centrifugation. Non-radioactive cytokinin analogues were added from

concentrated (up to 10^{-1} M) stock solutions in dimethylformamide. The total dimethylformamide concentration in the binding assays was kept at or below 0.1% (v/v). Dimethylformamide at these concentrations did not affect the binding assay results.

Bound radioactivity was measured in an 80,000 x *g* resuspended pellet by centrifugation at 15,000 rpm (Beckman Preparative Ultracentrifuge, Type 19 rotor) for 3 hr, using 4 ml capacity polypropylene tubes containing 3.2 ml solution. Adapters made from Delrin were used to accommodate 72 tubes per centrifuge run (12 tubes per adapter, 6 adapters per rotor). To measure binding in a 170,000 x *g* particulate fraction (Table 8), the assay solutions (3.2 ml) were centrifuged at 50,000 rpm for 30 min in a Beckman Preparative Ultracentrifuge (SW-56 rotor) using 4 ml polyallomer tubes.

Centrifuge tubes made of cellulose nitrate and Millipore filters were found unsuitable for binding assays because of a high background of BA binding to the tubes and filters.

Bound radioactivity was determined by pouring off the supernatants, draining the tubes for 30 min, cutting off the bottom of the centrifuge tube containing the pellet and placing it into 10 ml scintillation fluid (4-0 g/l PPO(2,5-diphenyloxazole), 0.1 g/l dimethyl-POP(1,4-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene) in toluene, with 10% (w/v) BBS-3 Beckman Biosolv). The vials were agitated overnight on a horizontal shaker in the dark at room temperature. The next day, radioactivity was measured in a Packard Tri-Carb Scintillation Counter (Model 3375). This procedure resulted in the complete recovery of radioactivity bound in the pellets, as determined by comparison in one experiment with pellets in which the radioactivity was measured by combustion (Packard Tri-Carb Sample

Oxidizer Model 360). Counting efficiency was typically 30-35% for ^{3}H and 80-85% for ^{14}C and was monitored with each vial by automatic external standardization.

Binding terminology

"Saturable" binding is defined as the decrease in bound radioactivity caused by the addition of unlabelled cytokinin to the assay mixture. The assay tube with *p*-[^{3}H]-BA or 8-[^{14}C]-BA without unlabelled cytokinin is called the "A" tube. "B" tubes are in all respects identical to "A" tubes except for containing an excess of non-radioactive cytokinin. Thus, "saturable binding" is calculated by subtracting the radioactivity found in the pellet of the "B" tube from that in the pellet of the "A" tube. "Non-saturable binding" is defined as the radioactivity in the "B" tube pellet.

p-[^{3}H]-BA binding which is reduced by the presence of low concentrations ($<5 \times 10^{-7}$ M) of non-radioactive BA is called "*high*"-affinity saturable binding. This is in contrast to "*low*"-affinity saturable binding of *p*-[^{3}H]-BA or 8-[^{14}C]-BA, in which a reduction of the bound radioactivity requires the presence of still higher concentrations ($>5 \times 10^{-7}$ M) of non-radioactive BA.

The terms "*high*"- and "*low*"-affinity binding are meant to distinguish binding sites with different K_d's, which usually reflect differences in binding affinity.

Protein and RNA determinations

Protein was measured according to Lowry et al. (1951), and RNA by the Schmidt-Thannhauser procedure, according to Fleck and Munro (1962).

Enzyme assays

To measure the activity of cytochrome c oxidase, a mitochondrial marker enzyme, sample aliquots (5-50 µl) were added to 0.4 ml reaction buffer (50 mM tris-acetate, pH 7.5) containing 0.1% Triton X-100. After 5 min at room temperature, the reaction was started by adding 50 µl from a stock solution of bovine heart cytochrome c (5.4 mg/ml in reaction buffer) which had been reduced with sodium dithionite ($5 < A_{550}/A_{565} < 12$). Enzyme activity was calculated from the initial rate of increase in A_{550} using 1 ml cuvettes (1 cm pathlength) in a Gilford Model 240 Spectrophotometer.

The activities of glucan synthetase I (UDP-glucose-1,4-glucan-glucosyl transferase) and glucan synthetase II (UDP-glucose-1,3-glucanglucosyl transferase) were measured using a procedure developed by Ray, Hertel and their co-workers and described by Dohrmann (1975). According to these authors, glucan synthetase I activity in corn coleoptile homogenates is associated with a membrane fraction which bands at 30% sucrose (w/w) and which probably consists mainly of the Golgi apparatus, while glucan synthetase II activity peaks at 35-38% sucrose (w/w) and probably is associated with a plasma membrane fraction. The technique for determining glucan synthetase activities is based on measuring the incorporation of [^{14}C]-UDPG into alcohol-insoluble material. For the experiment with tobacco cell homogenates (Figure 25), 50 µl sample aliquots were added to 50 µl reaction buffer (containing 60 mM tris-acetate, pH 8.0 with either 130 mM $MgCl_2$ for glucan synthetase I assays or 2 mM non-radioactive UDPG for glucan synthetase II assays). The reaction was initiated by the addition of 20 µl [^{14}C]-UDPG in H_2O (90,000 cpm, 240 mCi/mmol). After 1 hr at 25°C, 50 µl of 0.1 M $MgCl_2$ was added for glucan synthetase I assays and 150

µl of 0.1 M $MgCl_2$ was added for glucan synthetase II assays, and the reaction was terminated by heating at 100°C for 1 min. Approximately 5 mg of carrier protein (using a pre-boiled 13,000 x *g* particulate fraction) was added and the radioactive reaction product was precipitated with 2 ml 70% ethanol and washed 3 times by centrifugation (Sorvall Centrifuge, SS-34 rotor, 5,000 x *g*) and resuspension in 2 ml 70% ethanol. After the final rinse, the pellet was resuspended in 10 ml scintillation fluid and radioactivity determined as above.

Media and radiochemicals

The grinding medium contained 0.25 M sucrose, 50 mM tris, 1 mM Na_2-EDTA, 0.1 mM $MgCl_2$, pH 7.9; the binding assay medium contained 0.25 M sucrose, 10 mM sodium citrate, 5 mM $MgSO_4$, 0.5 mM $MgCl_2$, pH 6.0; the gradient medium (Figure 25, Ia and Ib) contained 10 mM tris, 1 mM Na_2-EDTA, 0.1 mM $MgCl_2$, 1 mM KCl, pH 7.0. The pH of all media was adjusted at room temperature with glacial acetic acid. One millimolar potassium metabisulfite was added to the grinding medium the same day, or one day before use.

8-[^{14}C]-BA (24 mCi/mmol) and 8-[^{14}C]-adenine (54 mCi/mmol) were obtained from Amersham Radiochemical Centre. [^{14}C]-UDPG (uniformly labelled, 240 mCi/mmol) was obtained from New England Nuclear. *p*-[^{3}H]-BA (10 Ci/mmol) was prepared as described (Section I, and Sussman and Firn, 1976).

Results

Comparison of the binding of 8-[^{14}C]-BA and *p*-[^{3}H]-BA

In a typical experiment in which 8-[^{14}C]-BA binding was measured *in vitro* to an 80,000 x *g* particulate fraction of tobacco cells

(Table 4, third column), of a total of 2457 dpm [^{14}C]-BA bound, 517 dpm (21%) was saturable. Thus, 1,940 dpm (79%) [^{14}C]-BA was non-saturably bound, i.e., was bound in the pellet even in the presence of 10^{-4} M non-radioactive BA. Saturability at higher concentrations of non-radioactive BA could not be tested since its solubility limit in H_2O lies at 1-2 x 10^{-4} M.

In a separate experiment, 8-[^{14}C]-adenine was used to estimate the amount of radioactivity which was simply trapped in the pellet because of the volume of free water. Radioactive adenine was chosen for this purpose because [^{12}C]-adenine did not compete for 8-[^{14}C]-BA binding and neither [^{12}C]-adenine nor [^{12}C]-benzyladenine competed for 8-[^{14}C]-adenine binding. Using 8-[^{14}C]-adenine it was found that only 27% of the non-saturably bound 8-[^{14}C]-BA could be accounted for by the trapped water of the pellet. This value is an upper limit since it assumes that there is no binding of 8-[^{14}C]-adenine by the pellet beyond that trapped in the free water.

The binding properties of *p*-[^{3}H]-BA at high (10 Ci/mmol) and low (24 mCi/mmol) specific activities were compared in the same experiment with that obtained with 8-[^{14}C]-BA (26 mCi/mmol). The results (Table 4) show that identical values were obtained for saturable binding when *p*-[^{3}H]-BA and 8-[^{14}C]-BA were used at the same specific activity and concentration. Results with the two isotopes were also identical when the total level of saturable binding was changed by tissue pre-treatment such as heat or 1 M KCl. However, the non-saturable binding was consistently higher when *p*-[^{3}H]-BA was used as compared to 8-[^{14}C]-BA at the same specific activity and concentration.

Table 4. Comparison of 8-[^{14}C]-BA and *p*-[^{3}H]-BA in binding assays using an 80,000 x *g* particulate fraction from tobacco cells

Radioactive tracer:		*p*-[^{3}H]-BA	*p*-[^{3}H]-BA	8-[^{14}C]-BA
Specific activity in "A" tube:		10 Ci/mmol	26 mCi/mmol	24 mCi/mmol
BA concentration in "A" tube:		4×10^{-9} M	2×10^{-6} M	2×10^{-6} M
BA concentration in "B" tube:		2×10^{-7} M	10^{-4} M	10^{-4} M
Type of saturable binding observable:		*high*-affinity	*low*-affinity	*low*-affinity
Pre-treatment		dpm bound per pellet ± S.E. (% of control)		
Control	A	7,240 ± 88	5,120 ± 47	2,457 ± 28
	B	5,478 ± 81	4,646 ± 139	1,940 ± 17
	A-B	1,762 ± 119	474 ± 164	517 ± 32
100°C, 15 min	A	9,186 ± 77	7,537 ± 129	4,145 ± 37
	B	7,804 ± 187 (142%)	6,403 ± 76 (138%)	3,062 ± 8 (158%)
	A-B	1,382 ± 191 (78%)	1,134 ± 204 (239%)	1,083 ± 37 (209%)
1 M KCl	A	9,143 ± 34	6,930 ± 203	3,429 ± 60
	B	7,361 ± 114 (134%)	6,050 ± 34 (130%)	2,593 ± 19 (133%)
	A-B	1,782 ± 119 (100%)	881 ± 249 (185%)	836 ± 63 (161%)

High- and *low*-affinity saturable BA binding

Low-affinity saturable binding was observed to increase in proportion to the increase in non-saturable binding after pre-treatment (Table 4). Thus, when the non-saturable binding rose to 158% and 133% of control, because of a pre-treatment with heat or 1 M KCl, respectively, the *low*-affinity saturable binding was also observed to increase, to 209% and 161% of controls, respectively. Similarly, when the non-saturable binding of 8-[^{14}C]-BA decreased, such as after a pronase or Triton X-100 pre-treatment, the *low*-affinity saturable binding also decreased to approximately the same extent.

In contrast to these results, *high*-affinity saturable binding, measurable only at the low concentrations possible with *p*-[^{3}H]-BA (Table 4), was unchanged by a 1 M KCl pre-treatment and decreased to 78% of controls following heating. In the same experiment, the non-saturable binding observed in the presence of 2×10^{-7} M non-radioactive BA and 4×10^{-9} M *p*-[^{3}H]-BA was increased to a similar extent (142% and 134% for pre-heating and 1 M KCl, respectively) as that observed for the *low*-affinity saturable and non-saturable binding.

In order to examine in greater detail the effects of heat on *high*- and *low*-affinity, saturable binding and to obtain estimates for the K_d's and R_t's (total number of binding sites), binding was measured over a range of BA concentrations. The raw data obtained (Table 5) confirm the previous results, in that the heat lability is a property only of the saturable binding which is observed when the "B" tube contains low concentrations of non-radioactive BA. With the "A" tube at 6.2 nM *p*-[^{3}H]-BA and the concentration of non-radioactive BA in the "B" tube increasing from 17 nM to 862 nM, there is a progressive

Table 5. Concentration dependence of heat-labile, *high*-affinity cytokinin binding to an 80,000 x *g* particulate fraction from tobacco cells

Concentration[a] ^{1}H-BA added	Total Bound Radioactivity cpm per pellet		Saturably Bound Radioactivity cpm per pellet		% of Saturable binding which is heat-labile
	Control	Pre-heated	Control	Pre-heated	
-	7411 ± 51	8695 ± 33	-	-	-
17 nM	6889 ± 113	8858 ± 18	522 ± 124	-163 ± 38	100
43 nM	6834 ± 53	8589 ± 62	577 ± 74	106 ± 94	84
129 nM	6546 ± 64	8523 ± 41	865 ± 82	172 ± 61	83
216 nM	6418 ± 68	8527 ± 58	933 ± 85	168 ± 67	85
431 nM	6238 ± 19	8327 ± 106	1173 ± 54	368 ± 111	76
862 nM	5928 ± 33	7920 ± 138	1483 ± 61	775 ± 142	66

[a]All samples contained 6.2 nM *p*-[^{3}H]-BA (10 Ci/mmol).

decrease in the percent of the saturable binding which is heat-labile, from 100% to 66%.

When the data for binding in the control samples are plotted according to Scatchard (1949), a non-linear relation is obtained (Figure 22) which can be resolved into two straight lines, representing the equilibrium binding parameters for *high*- and *low*-affinity binding sites. In contrast, the Scatchard plot for binding in a pre-heated sample clearly demonstrates the absence of the *high*-affinity binding component. These data for the pre-heated sample were fitted to a single straight line by least squares analysis with a high correlation coefficient ($r = -0.964$). Another effect of the heat pre-treatment, which is evident from this plot, is a 10% *increase* in the number of *low*-affinity binding sites, with no effect on the K_d. A graphical technique (Rosenthal, 1967) to obtain precise values for the *high*-affinity binding parameters was found unsuitable, due to the fact that the *high*-affinity binding accounts for at most only about 10-15% of the total bound/free. Thus, the values shown in Figure 22 ($K_d = 1.4 \times 10^{-7}$ M, $R_t = 8 \times 10^{-9}$ mol/Kg fresh weight) were calculated by visually estimating a straight line parallel to the steepest part of the *high*-affinity portion of the control binding curve. Because of interference by the large excess of *low*-affinity binding sites, the K_d and R_t values for the *high*-affinity site are probably upper limits and should only be considered as estimates of the true values.

Structure-activity requirements of *in vitro* ^{3}H-BA binding

(i) 80,000 x *g* particulate fraction from tobacco cells. The ability of the cytokinin analogues to compete with *p*-[^{3}H]-BA at the

Figure 22. Scatchard plot of *p*-[^{3}H]-BA binding at different BA concentrations to show *high*-affinity and *low*-affinity cytokinin binding sites in an 80,000 x *g* particulate fraction from tobacco cells and their contrasting sensitivity to a heat pre-treatment (100°C, 15 min).

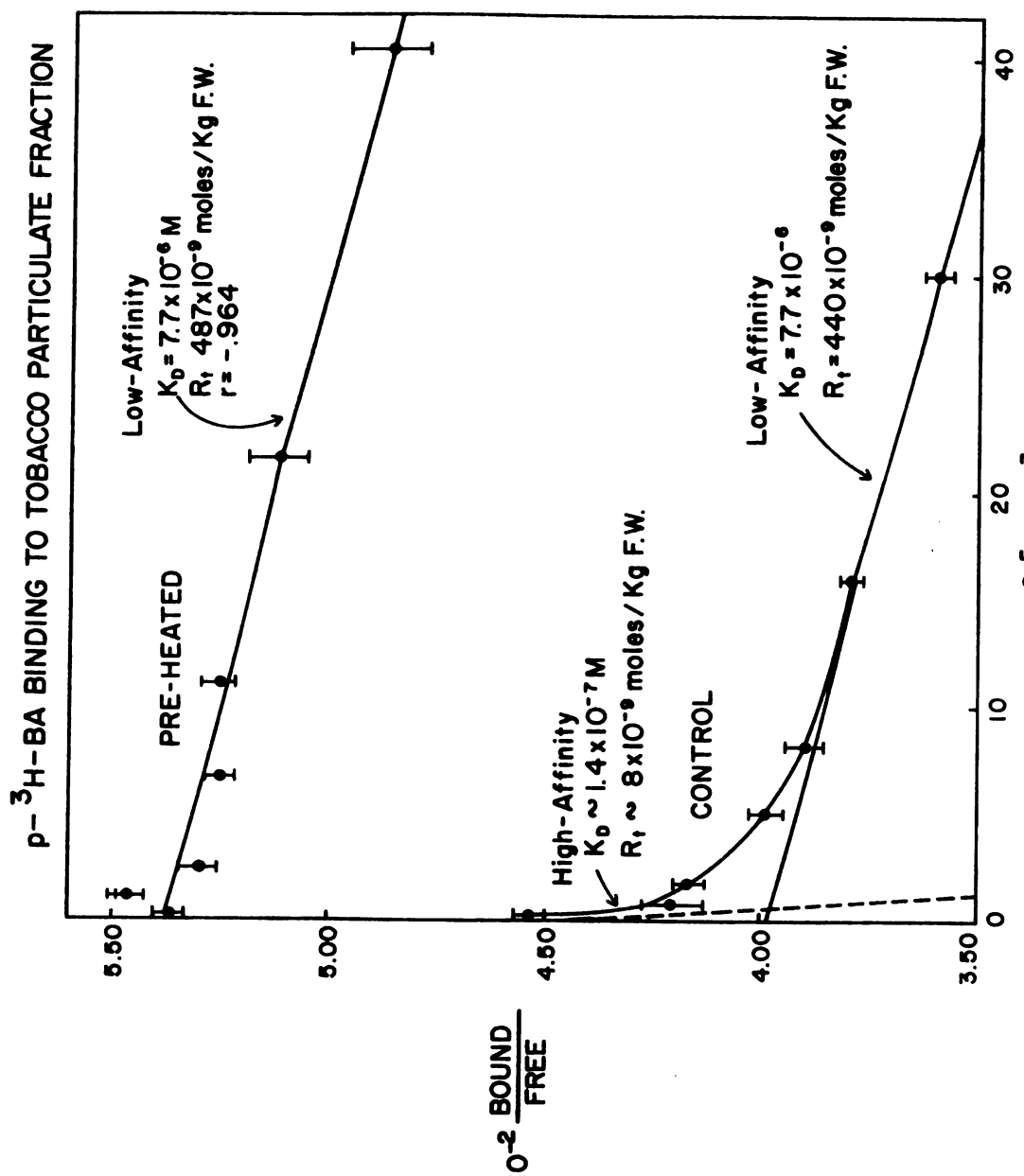

p-³H-BA BINDING TO TOBACCO PARTICULATE FRACTION
PRE-HEATED
Low-Affinity
K_D = 7.7x10⁻⁶ M
R_t 487x10⁻⁹ moles/Kg F.W.
r² = -.964
High-Affinity
K_D ~ 1.4x10⁻⁷M
R_t ~ 8x10⁻⁹ moles/Kg F.W.
CONTROL
Low-Affinity
K_D = 7.7x10⁻⁶
R_t = 440x10⁻⁹ moles/Kg F.W.
10⁻² BOUND/FREE
10⁻⁹ [BOUND] (M)
5.50
5.00
4.50
4.00
3.50
0
10
20
30
40

Figure 22

high-affinity binding site was measured (Table 6). The order of their activity was: BA > *o*-F-BA = *p*-F-BA = kinetin > *m*-F-BA > *o*-Cl-BA > *o*-Br-BA > *trans*-zeatin > *cis*-zeatin > *m*-Cl-BA > *p*-Cl-BA > *p*-Br-BA. This relative order of binding activity closely parallels that observed for the biological activity of these analogues in the same tissue (Figure 17).

When the ability of the cytokinin analogues to compete with *p*-[^{3}H]-BA at the *low*-affinity binding site was measured, all of the analogues showed approximately equal activity as BA, except for *o*-F-BA and *p*-F-BA, which exhibited slightly higher activity (Table 7).

(ii) Talc. *p*-[^{3}H]-BA was found to bind saturably to talc powder (Mallinkrodt, U.S.P.) in a manner superficially resembling that which we observed with a particulate fraction from tobacco cells. In a Scatchard plot (Figure 23), *p*-[^{3}H]-BA binding to talc gave a non-linear curve which was visually resolved into two straight lines representing *high*-affinity (K_d = 4.5 x 10^{-8} M, R_t = 1.3 x 10^{-8} mol/Kg dry talc) and *low*-affinity (K_d = 2.3 x 10^{-6} M, R_t = 2.4 x 10^{-7} mol/Kg dry talc) binding sites. The *low*-affinity saturable binding was found to be non-specific in its structural requirements since all of the cytokinin analogues tested competed approximately equally well (Table 8). In contrast, the analogues showed differing abilities to compete for the *high*-affinity saturable binding and their observed order was: *p*-Br-BA = *o*-Br-BA = *o*-Cl-BA > *m*-Cl-BA > *p*-Cl-BA > *p*-F-BA = BA > *o*-F-BA = *m*-F-BA. This is totally unlike the specificity obtained in any bioassay and biological binding test.

Table 6. Analogue specificity of *high*-affinity saturable cytokinin binding in an 80,000 x *g* particulate fraction from tobacco cells

Compound tested[a]	Saturably bound radioactivity cpm per pellet ± S.E.
BA	318 ± 27
o-F-BA	279 ± 20
m-F-BA	249 ± 26
p-F-BA	271 ± 27
o-Cl-BA	227 ± 25
m-Cl-BA	118 ± 34
p-Cl-BA	72 ± 28
o-Br-BA	200 ± 32
p-Br-BA	34 ± 24
Kinetin	284 ± 27
cis-Zeatin	134 ± 22
trans-Zeatin	166 ± 16

[a]All tubes contained 5 nM *p*-[^{3}H]-BA (10 Ci/mmol in "A" tubes). The non-radioactive compounds tested above were added to "B" tubes at 500 nM.

Table 7. Analogue specificity of *low*-affinity saturable cytokinin binding in an 80,000 x *g* particulate fraction from tobacco cells

Compound tested[a]	Saturably bound radioactivity cpm per pellet ± S.E.
BA	1053 ± 125
o-F-BA	1401 ± 104
m-F-BA	964 ± 21
p-F-BA	1411 ± 72
o-Cl-BA	942 ± 65
m-Cl-BA	1187 ± 187
p-Cl-BA	1047 ± 130
o-Br-BA	1248 ± 159
p-Br-BA	969 ± 40

[a]All tubes contained 5 nM *p*-[^{3}H]-BA plus 1500 nM ^{1}H-BA (final specific activity of 400 mCi/mmol). The non-radioactive compounds tested above were added to "B" tubes at 10 μM.

Figure 23. Scatchard plot of *p*-[^{3}H]-BA binding to talc.

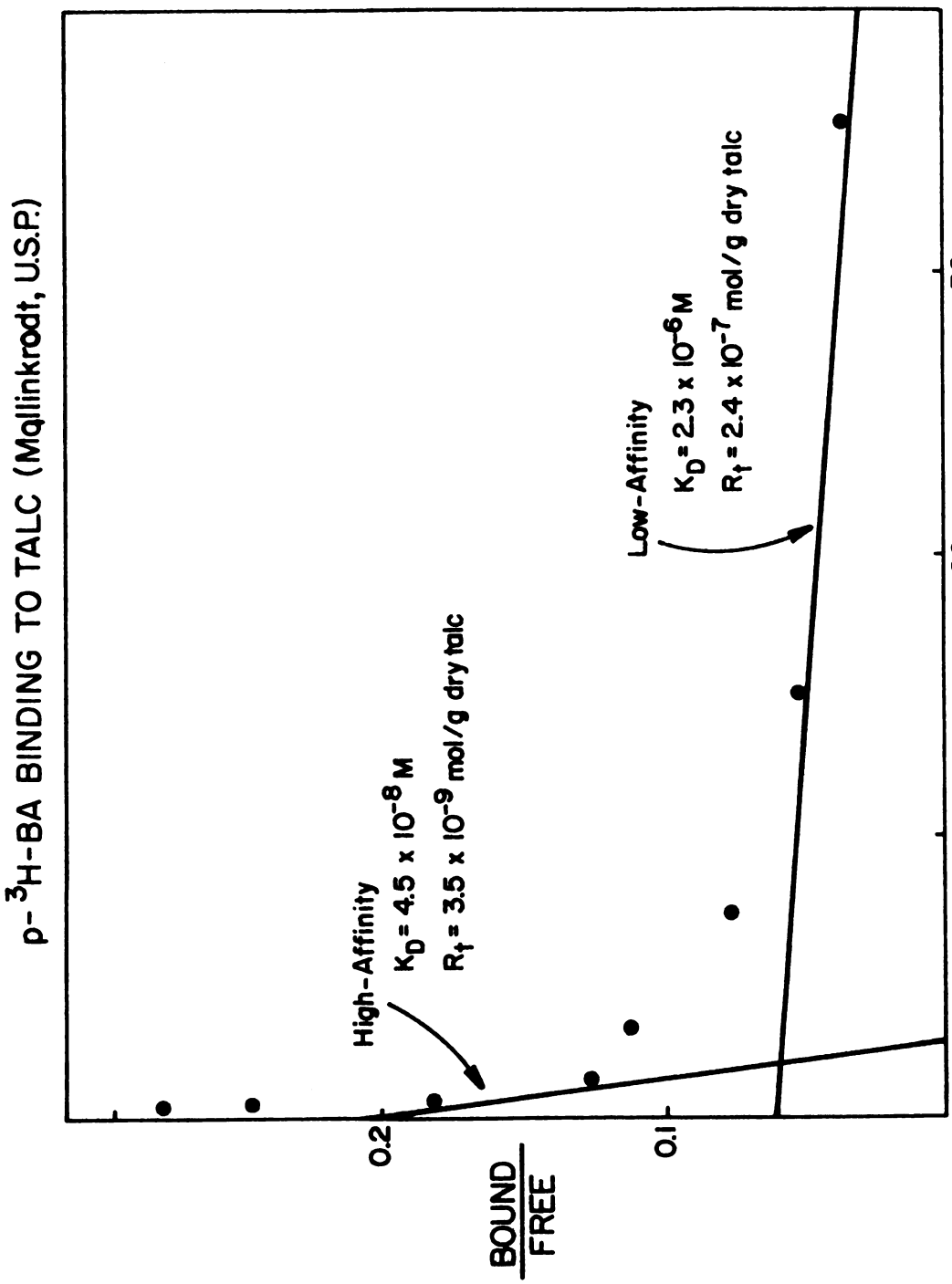

p-^{3}H-BA BINDING TO TALC (Mallinkrodt, U.S.P.)
High-Affinity
K_D = 4.5 x 10^{-8} M
R_t = 3.5 x 10^{-9} mol/g dry talc
Low-Affinity
K_D = 2.3 x 10^{-6} M
R_t = 2.4 x 10^{-7} mol/g dry talc
BOUND / FREE
0.2
0.1
10
20
30
10^{-9} [BOUND] (M)

Figure 23

Table 8. Analogue specificity of *high*- and *low*-affinity saturable cytokinin binding to talc (Mallinkrodt, U.S.P.)

Compound tested	Saturably bound radioactivity cpm per pellet + S.E.	
	Low-affinity[a]	*High*-affinity[b]
BA	1002 ± 45	873 ± 180
o-F-BA	956 ± 43	566 ± 41
m-F-BA	989 ± 31	495 ± 49
p-F-BA	1030 ± 30	872 ± 173
o-Cl-BA	1111 ± 31	1480 ± 71
m-Cl-BA	1135 ± 28	1271 ± 243
p-Cl-BA	1143 ± 40	1092 ± 40
o-Br-BA	1158 ± 32	1461 ± 239
p-Br-BA	1147 ± 32	1430 ± 42

[a] *Low*-affinity saturable binding was calculated using "A" tubes with 0.5 nM *p*-[^{3}H]-BA + 100 nM ^{1}H-BA and "B" tubes containing 0.5 nM *p*-[^{3}H]-BA + 100 nM ^{1}H-BA + 10 µM non-radioactive analogue.

[b] *High*-affinity saturable binding was calculated using "A" tubes containing 0.5 nM *p*-[^{3}H]-BA and "B" tubes with 0.5 nM *p*-[^{3}H]-BA + 100 nM non-radioactive analogue.

Localization of the *high*-affinity cytokinin binding site from tobacco cells

(i) Differential centrifugation. Recently Fox and Erion (1975) reported that ribosomes isolated from wheat germ possess a specific cytokinin binding protein. In order to test whether the *high*-affinity cytokinin binding site which I had detected in particulate cell-free fractions of tobacco cells was associated with ribosomes, various fractions enriched in ribosomes were obtained by differential centrifugation. It was found (Table 9) that on a per mg protein basis, RNA was enriched in the low-speed (10,000-13,000 x *g*) and high-speed (80,000-170,000 x *g*) fractions, presumably because of the presence in these fractions of membrane-bound and free ribosomes, respectively (Davies and Larkins, 1975). In contrast to the observed distribution of ribosomes, *high*-affinity, heat-labile, saturable cytokinin binding activity was found to be enriched in the intermediate-speed (13,000-80,000 x *g*) fraction.

Fox and Erion (1975) also reported that the ribosomal cytokinin-binding protein from wheat germ could be solubilized by a wash with 0.5 M KCl. In contrast, I found that there was no reproducible, significant effect of 1 M KCl on *high*-affinity saturable cytokinin binding in particulate fractions from tobacco cells (Table 4, and also Table 8). In this centrifugation assay, solubilization would be observed as a reduction in the saturably bound radioactivity, which did not occur.

(ii) Isopycnic sucrose density gradient centrifugation. Using fractions obtained by isopycnic sucrose density gradient centrifugation of a homogenate from tobacco cells, an attempt was made to

Table 9. Comparison of cytokinin-binding activity, RNA and protein content in various tobacco cell particulate fractions obtained by differential centrifugation

Fraction	Pre-treatment	Protein mg/ pellet[b]	RNA mg/ pellet[b]	RNA mg/ mg protein	*High*-Affinity Saturable Binding[a] cpm/ pellet[b]	cpm/ mg protein	heat-labile cpm/ mg protein
0-13,000 x *g*	Control	1.35	0.123	0.091	2,012 ± 217	1,490 ± 161	
	100°C, 15 min	1.10	-	-	905 ± 110	838 ± 100	652 ± 190
	1 M KCl	1.12	-	-	1,235 ± 168	1,098 ± 150	
13,000 x *g* - 80,000 x *g*	Control	0.82	0.066	0.080	1,385 ± 135	1,679 ± 165	
	100°C, 15 min	0.60	-	-	429 ± 54	715 ± 90	964 ± 187
	1 M KCl	0.62	-	-	1,373 ± 235	2,034 ± 379	
80,000 x *g* - 170,000 x *g*	Control	0.18	0.018	0.100	154 ± 45	856 ± 250	
	100°C, 15 min	0.16	-	-	89 ± 57	540 ± 356	316 ± 435
	1 M KCl	0.12	-	-	138 ± 56	1,150 ± 467	

[a]All samples contained 5.3 nM *p*-[^{3}H]-BA (10 Ci/mmol in "A" tubes); 860 nM non-radioactive BA was added to "B" tubes.

[b]Each pellet corresponded to 2.2 g fresh weight of tobacco cells.

determine whether specific binding was associated with a particular membrane or organelle (Figure 24).

Membrane marker enzymes were used to locate the Golgi apparatus (peak of glucan synthetase I activity at 35% (w/w) sucrose), a plasma membrane fraction (peak of glucan synthetase II activity at approximately 39-40% (w/w) sucrose), and mitochondria (peak of cytochrome c oxidase at 41-42% (w/w) sucrose).

Visual examination of the centrifuge tubes following sucrose gradient centrifugation (Figure 24) consistently showed 4 distinct bands. Since bands 2 and 4 were the only green bands and band 4 was more prominent when less harsh grinding conditions were used (i.e., grinding by hand with a porcelain mortar and pestle instead of the Super Dispax Tissumizer), these fractions were identified as containing stripped (Band 2) and intact (Band 4) chloroplasts (Miflin and Beevers, 1974).

A marker enzyme for smooth endoplasmic reticulum was not measured, but this membrane was tentatively assigned to band 1 since it is the only prominent lower density visual band and a similar highly turbid zone which is considered to be smooth endoplasmic reticulum is found in gradients of corn homogenates (R. Hertel, personal communication). Band 3 was the most highly concentrated, intense visual band and was identified as containing mitochondria because it coincided with the peak of cytochrome c oxidase activity.

Because the enzyme assays required only low quantities of sample, many fractions could be collected and tested for activity, allowing finer resolution of overlapping peaks (Figure 24, II). In contrast, resolution was greatly sacrificed in the fractions obtained for the cytokinin binding assay (Figure 24, Ia and Ib). In order to

Figure 24. Visually observed bands in linear sucrose density gradients after centrifugation of an homogenate from tobacco cells.

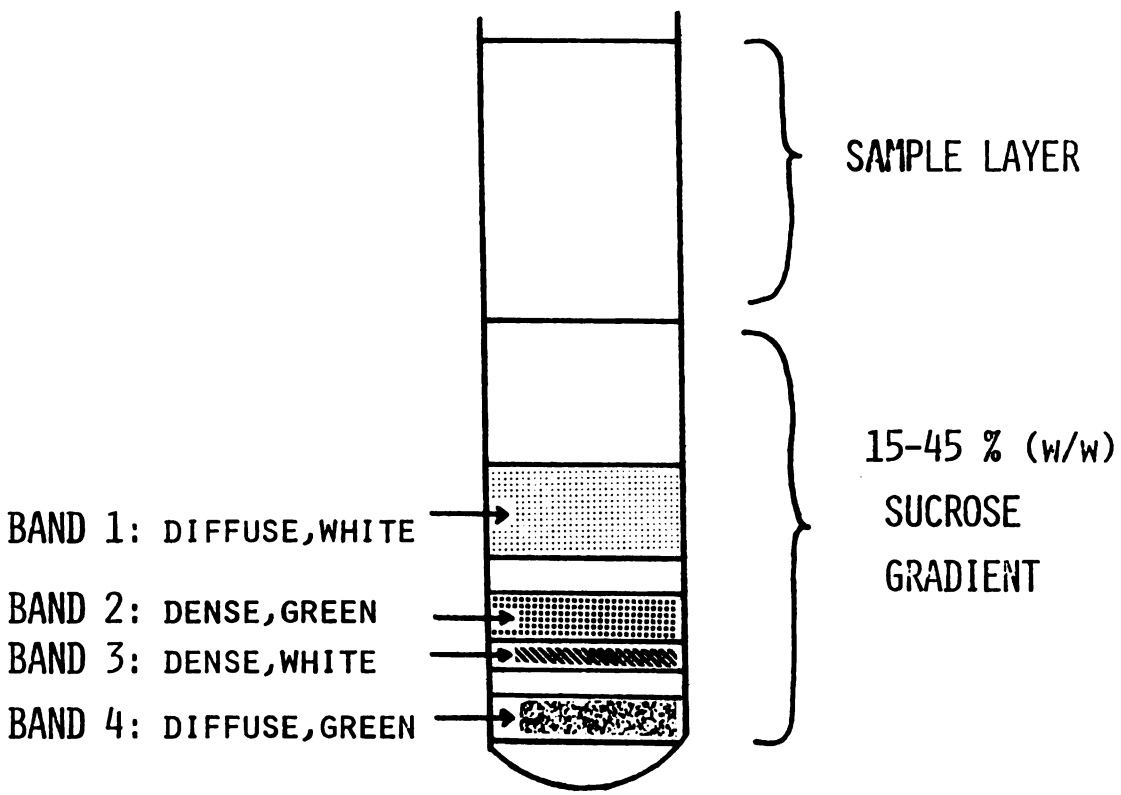

SAMPLE LAYER
15-45 % (w/w)
SUCROSE
GRADIENT
BAND 1: DIFFUSE,WHITE
BAND 2: DENSE,GREEN
BAND 3: DENSE,WHITE
BAND 4: DIFFUSE,GREEN

Figure 24

obtain sufficient cell material for the 12 binding assay tubes which were needed for each fraction (3 "A" tubes and 3 "B" tubes for a control sample and 3 "A" tubes and 3 "B" tubes for a pre-heated sample), it was necessary to (1) collect a smaller number (six) of fractions with larger volumes from each gradient and (2) pool the fractions collected from six gradients. The problems of processing sufficient biological material is further aggravated by the background of heat-stable, *low*-affinity cytokinin binding. These experiments were originally attempted to improve the signal-to-noise ratio based on the possibility that *high*-affinity binding might reside exclusively in a membrane fraction readily separable from the bulk of all others. Unfortunately, as detailed below, this did not prove to be the case.

Despite the difficulties, the following limited conclusions may be derived from the data. The level of non-saturable binding closely followed the protein content of each fraction (Figure 25, Ib) and was observed, as in all other experiments, to increase following a heat pre-treatment. *High*-affinity, saturable binding was also most prominent in the fractions containing the highest protein content. In this particular experiment (Figure 24, Ia), fractions #4 and #5 were the only ones containing heat-labile, *high*-affinity saturable binding sites. Of two separate repeats of this experiment, one yielded similar results and the other showed heat-labile, *high*-affinity saturable binding only in fractions #5 and #6. Thus, fractions #4 and #6 showed variable amounts of heat-labile, *high*-affinity saturable binding, while fraction #5 most consistently exhibited this activity.

Figure 25. Fractionation of membrane marker enzymes and BA binding activity after centrifugation of a tobacco cell homogenate on linear sucrose density gradients. The results in Ia and Ib were obtained with aliquots from the same pooled gradient fractions in one experiment. The results in II were obtained with a gradient in a separate experiment. All conditions in II were the same as in Ia and Ib (see Materials and Methods for media) except that the grinding medium contained 50 mM MES (2(N-morpholino)ethane sulfonic acid)-NaOH, pH 6.0 instead of 50 mM tris-NaOH, pH 7.9, and 0.1 mM $MgCl_2$ was deleted. The gradient medium contained 10 mM MES-NaOH, pH 6.0 instead of 10 mM tris-NaOH, pH 7.0, and the 0.1 mM $MgCl_2$ and 1 mM KCl were deleted. In all experiments, 17 ml of a Miracloth filtrate obtained from a tobacco cell homogenate was layered onto 21.5 ml of a linear 15-45% (w/w) sucrose gradient and centrifuged in a Beckman Preparative Ultracentrifuge (SW-27 rotor, 27,000 rpm) for 3 hr at 95,000 x *g*. Fractions were collected by tube puncture and gravity-feed, from the bottom.

In Ia, ●——● is sucrose concentration [% w/w]; Δ——Δ and ▲——▲ represent *high*-affinity saturable BA binding in a control and pre-heated (100°C, 15 min) aliquot of the fraction, respectively. In Ib, ●····● is protein; Δ——Δ and ▲---▲ represent non-saturable BA binding (5 nM *p*-[^{3}H]-BA in "A" and "B" tubes, 100 nM non-radioactive BA in "B" tubes) in control and pre-heated samples, respectively.

In II, ●——● is % sucrose concentration [% w/w], o---o is glucan synthetase I activity, x——x is glucan synthetase II activity and ▲···▲ is cytochrome c oxidase activity.

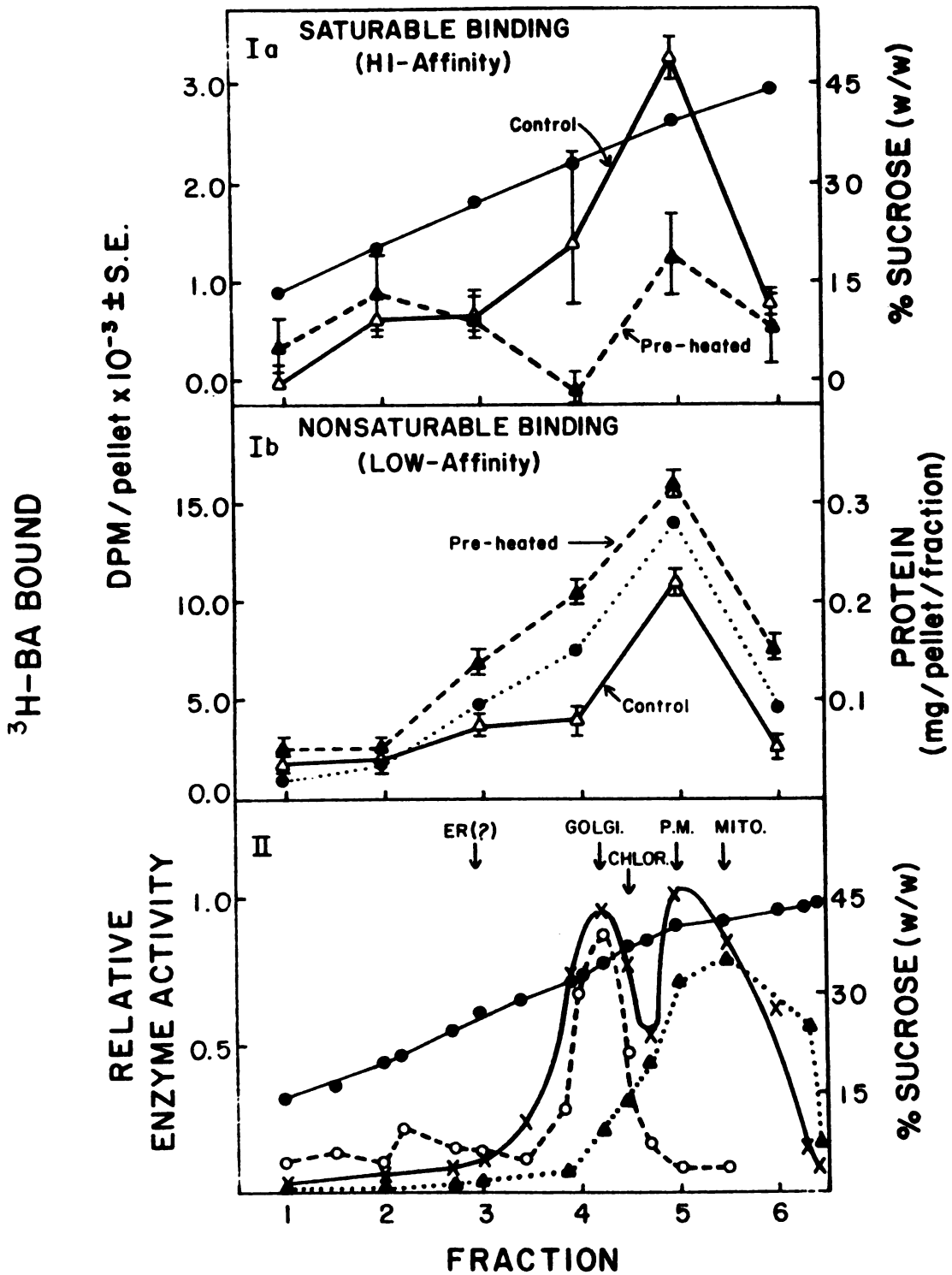

Ia
SATURABLE BINDING
(HI-Affinity)
Control
Pre-heated
Ib
NONSATURABLE BINDING
(LOW-Affinity)
Pre-heated
Control
II
ER(?)
GOLGI.
CHLOR.
P.M.
MITO.
^{3}H-BA BOUND
DPM/pellet x 10^{-3} ± S.E.
% SUCROSE (w/w)
PROTEIN (mg/pellet/fraction)
RELATIVE ENZYME ACTIVITY
FRACTION

Figure 25

Discussion

Low-affinity binding as measured with 8-[^{14}C]-BA and *p*-[^{3}H]-BA

Binding assays with benzyladenine labelled with ^{3}H or ^{14}C at the same low specific activity gave identical results for saturable binding, but not for non-saturable binding. In all treatments, there was approximately twice as much *p*-[^{3}H]-BA non-saturably bound than 8-[^{14}C]-BA.

There are two possible explanations for this result: (1) the higher level of *p*-[^{3}H]-BA binding was due to a radioactive impurity in the sample or (2) the difference was caused by an isotope effect. The *p*-[^{3}H]-BA sample used had been purified by chromatography on both cellulose (Figure 4) and Sephadex LH-20 (Figure 5), and re-chromatography in these and other systems (see Section I) showed a radiochemical purity of 99%. If there was a radiochemical impurity in the *p*-[^{3}H]-BA sample, it must either be chromatographically very similar to BA (and have a similar UV absorption spectrum) or else it must be present at very low radioactive concentrations.

Since this extra binding of *p*-[^{3}H]-BA was totally non-saturable (i.e., it occurred even in the presence of 10^{-4} M BA), it did not interfere with the measurement or interpretation of saturable cytokinin binding. In other words, its only effect was to increase (double) the background "noise" without affecting the signal.

Specific versus non-specific BA binding

Only the *high*-affinity saturable *p*-[^{3}H]-BA binding which I observed in a particulate fraction from tobacco cells fulfills the most important criterion which characterizes "specific" binding;

that is, the structural requirements for binding were identical to those observed in the biological response. The conclusion that this binding was specific is supported by the contrasting lack of specificity for *low*-affinity binding with this fraction from tobacco cells and also by the observation that genuinely artifactual binding to a non-biological material (talc) had a structural specificity totally unlike the specificity of the biological binding and bioassay results.

The observation that only the *high*-affinity, saturable binding was heat-denaturable is additional evidence that only this binding site resides in a specific protein.

In contrast, a heat pre-treatment caused a 10% *increase* in the number of *low*-affinity saturable binding sites. A similar observation was reported by Puca et al. (1971) for the non-specific binding of estrogen by bovine plasma albumin. These workers interpreted this increase in non-specific binding as due to a loss of protein conformation, with a resultant increase in protein surface and, thus, in non-specific binding sites. This explanation may also hold for the heat-induced increase in *low*-affinity saturable binding which we observed in fractions from tobacco cell.

Localization of specific cytokinin binding site

In the assay for specific cytokinin binding, the signal was low, as was the signal-to-noise ratio. For this reason, it was obviously difficult to determine precisely with which cell structure this binding site was associated. In spite of these difficulties, I conclude from results which were obtained consistently that it is associated with a structure which is present in all fractions obtained by differential centrifugation, but is enriched in a 13,000-80,000 x *g*

particulate fraction. This structure also bands at a density corresponding to 35-42% (w/w) sucrose under conditions at which equilibrium sedimentation of cell membranes and organelles is obtained (Hodges and Leonard, 1973).

The results on insensitivity of specific binding to 1 M KCl indicate the *high*-affinity binding site which I have detected in a particulate fraction from tobacco cells is unlike that obtained by Fox and Erion (1975) from ribosomes of wheat germ. My differential centrifugation studies further suggest that this binding site is not associated with ribosomes; however, since the differences in specific binding activity between the particulate fractions were barely significant, these conclusions remain tentative.

Finally, because the *high*-affinity binding site was found to be associated with a structure which banded in equilibrium sucrose gradients in the vicinity of the bulk of the cell organelles and membranes, it was not possible to determine which of these structures were responsible for the binding activity.

Where do we go from here?

At this point, it is clear that a more conclusive localization of the *high*-affinity, heat-labile cytokinin binding site will require an increase in the signal-to-noise ratio generated in the binding assay. This may be obtained by including 10^{-5} M *p*-Br-BA in all of the binding assay mixtures since this compound was observed to compete well for the *low*-affinity, non-specific binding site but not at all for the *high*-affinity binding site in particulate fractions from tobacco cells. A further increase in the signal-to-noise ratio may be obtained by increasing at least 10-fold the amount of biological

material over that used in this study. The tobacco cells which I have used are easily grown to high densities, and there would be no problem in obtaining sufficient cells. However, for sucrose density gradient studies with larger amounts of material, it would be necessary to use a zonal rotor (e.g., the Sorvall sZ-14).

Another possible approach is to attempt to increase the signal and improve the signal-to-noise ratio by optimizing the extraction and assay conditions. This was not attempted in the present study beyond the initial, preliminary experiments. This approach was used successfully by Dohrman (1975) to extend the initial studies on an auxin binding site from corn coleoptiles (Hertel et al., 1972) and to more definitively characterize its localization, pH sensitivity, etc.

As an alternative approach, I synthesized a cytokinin derivative which has the capacity to *covalently* combine with its receptor. My work using this technique, known commonly as photoaffinity labelling, is described in the next section.

SECTION IV

CHEMICAL SYNTHESIS AND BIOLOGICAL ACTIVITY OF 8-AZIDO-BENZYLADENINE, A POTENTIAL CYTOKININ PHOTOAFFINITY REAGENT

Introduction

Carbene and nitrene generating, light-sensitive derivatives of hormones and substrates have proven useful to analyze in animal tissues hormone receptors and active sites of enzymes (see review by Knowles, 1972). Recently, Haley and co-workers described the use of radioactive 8-azido-substituted purine derivatives to covalently label ATP and cAMP binding proteins, including ATPases and a protein kinase (Haley and Hoffman, 1974; Haley, 1975; Pomerantz et al., 1975; Malkinson et al., 1975). A striking feature of these and other photoaffinity studies (Guthrow et al., 1973; Hanstein and Hotefi, 1974; Maassen and Moller, 1974) has been the ability to use the radioactive, light-sensitive derivatives with an impure, unfractionated cell-free extract to radioactively label only those proteins which have a specific binding site for the particular ligand.

I have undertaken the synthesis of a radioactive cytokinin photoaffinity derivative (8-N_3-BA) in the hope that, in combination with SDS gel electrophoresis, it will permit identification of a specific cytokinin-binding protein(s), as has been achieved in analogous studies with 8-azido-ATP and 8-azido-cAMP using extracts of animal tissues. In addition to the obvious advantages that a light-generated covalent bond between a hormone and its receptor has for analysis of

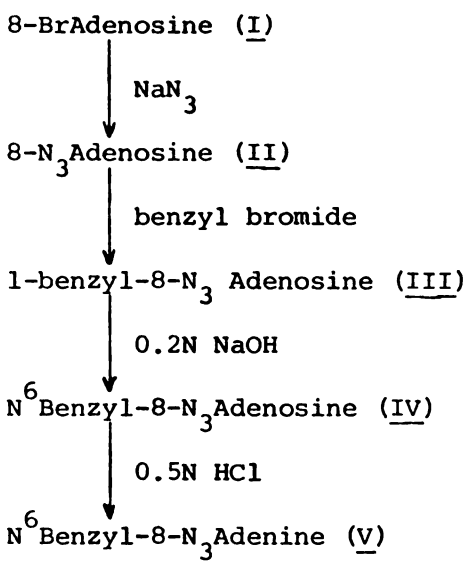
hormone binding *in vitro*, it may also be possible to perform the photolysis and covalent-bond formation in the intact tissue *prior* to cell rupture, as, for example, has been suggested to occur during the *in vitro* analysis of auxin-stimulated enzyme activity in the plasma membrane of coleoptiles (Cleland, 1975).

In this section, I describe the chemical synthesis and photolytic properties of non-radioactive 8-azido-benzyladenine, and also results of biological experiments which were conducted prefatory to its use as a radioactive compound.

Materials and Methods

Synthesis of non-radioactive 8-N_3-BA

Attempts to brominate BA directly in the 8-position, according to procedures used to obtain 8-bromo-cAMP (Muneyama et al., 1971) and 8-bromo-adenosine (Holmes and Robins, 1964) yielded only 2-bromo-benzyladenine, as identified by UV and NMR spectroscopy and mass spectrometry. 2-Bromo-BA did not undergo displacement reaction to form the azido derivative when heated with NaN_3 in dimethylformamide under conditions used to obtain 8-azido-cAMP (Muneyama et al., 1971), 8-azido-adenosine (Holmes and Robins, 1965) and 8-azido-AMP (Haley and Hoffman, 1974). We thus chose an alternative route for the preparation of 8-N_3-BA, based on the synthesis of N^6-substituted adenine derivatives from adenosine, according to the alkylation and rearrangement procedure described by Leonard and Fuji (1964):



8-Azido-adenosine (II) was synthesized from 8-bromo-adenosine (I) (Aldrich Chemical Co., Milwaukee, WI, USA) according to Holmes and Robins (1965). To 165 mg NaN_3 (3 mmol) in 50 ml dimethylformamide 692 mg of I (2 mmol) was added and the solution heated at 75°C. The progress of the reaction was monitored by measuring the UV absorption spectrum of the product (Table 10).

After 15 hr, when the reaction was completed, the solvent was evaporated *in vacuo* at 80°C and the product, II, was precipitated from the viscous residue by addition of 50 ml methylene chloride. The precipitate was washed with 2 ml distilled H_2O, dried *in vacuo* at 50°C, dissolved in 6 ml dimethylformamide, and 0.43 g benzyl bromide (2.5 mmol) were added to it. The reaction mixture was stirred and heated at 37°C, and the formation of III was followed by TLC (Table 11). An additional 0.29 g benzyl bromide (1.7 mmol) was added after 24 hr. By 48 hr, the reaction was complete and the solution was added to 40 ml 0.2 N NaOH. A brown, immiscible oily residue (presumably unreacted benzyl bromide) which formed immediately was removed by centrifugation. The supernatant was then heated at 85°C

Table 10. UV spectra of intermediates and related compounds used in the synthesis of 8-N_3-BA[a]

Compound	0.1 N HCl		Neutral pH		0.1 N NaOH	
	λ_{max}	λ_{min}	λ_{max}	λ_{min}	λ_{max}	λ_{min}
8-Br-Adenosine (I)	262	231	264 213	231	265	237
8-N_3-Adenosine (II)	281 206sh[b]	244	281 221	248	280	249
1-Benzyl-8-N_3-Adenosine (III)	282 212sh	248	282 325sh	255	282 325sh	255
N^6-Benzyl-8-N_3-Adenosine (IV)	290	250	289	255	289	254
N^6-Benzyl-8-N_3-Adenine (V)	298 230sh	250	290 221sh	254	293	256
Adenine	262	227	260 207	224	267 277sh	238
Adenosine	257	230	259	226	259	234
1-Benzyladenosine	258	235	259 267sh	232	259 267sh	237
N^6-Benzyladenosine (BA-riboside)	264	234	269 208	231	268	236
N^6-Benzyladenine (BA)	276	236	270 210	231	276 282sh	242

[a] All spectra were obtained in 50% ethanol.

[b] sh denotes a shoulder.

Table 11. Thin layer chromatography on silica gel of intermediates and related compounds used in the synthesis of 8-N_3-BA

Compound	R_f [a]	
8-Br-Adenosine (I)	0.44	
8-N_3-Adenosine (II)	0.44	
1-Benzyl-8-N_3-Adenosine (III)	0.31	
N^6-Benzyl-8-N_3-Adenosine (IV)	0.71	(0.38)
N^6-Benzyl-8-N_3-Adenine (V)	-	(0.69)
Adenosine	0.30	
Adenine	0.32	
8-Br-Adenine	0.44	
N^6-Benzyladenine (BA)	0.61	
N^6-Benzyladenosine (BA-riboside)	0.59	
1-Benzyladenosine	0.10	

[a]Developing solvent was 16% methanol in methylene chloride, except for the values in parentheses which represent the R_f in 8% methanol in methylene chloride.

to effect rearrangement to IV. The reaction was monitored by TLC and found to be complete within 45 min. Estimated yield (by UV spectroscopy) was 350 mg (0.88 mmol) of IV. Cleavage of the ribose to give the 8-azido-substituted free base (V) was achieved by the addition of 6 N HCl to the above, cooled reaction mixture, to give a final concentration of 0.5 N HCl. After 45 min at 80°C, the reaction was complete, as judged by TLC. The pH was adjusted to 4.5 with 1 N NaOH and the product, V, was separated from the reaction mixture by extraction with ethyl acetate. Forty-two milligrams (0.12 mmol) of chromatographically pure V was recovered following recrystallization from ethanol. The major loss in overall yield (6% of theoretical final yield from I) was thus in the last, acid hydrolysis, step.

The identity of the final compound (V) as 8-N_3-BA was confirmed by its mass spectrum (Figure 26) and by its photo-labile properties (see below).

Cytokinin bioassays

For measuring cytokinin activity, two bioassays were used: growth of a cytokinin-dependent tobacco cell suspension culture and bud formation in 2-week-old moss protonemata. Both assays have been described in Section II.

The reader will notice that the maximum fresh weight per flask attainable with BA (10 g) in the more recent dose-response experiment with the tobacco cells (Figure 29) is approximately two-thirds that shown (15 g) in Figure 17. The experiments of Figure 29 were performed in the summer and fall of 1976, and those of Figure 17 during spring and summer of 1975. In the period between, it was necessary to discard the liquid cell cultures due to contamination and to start

Figure 26. Mass spectrum of 8-N_3-BA.

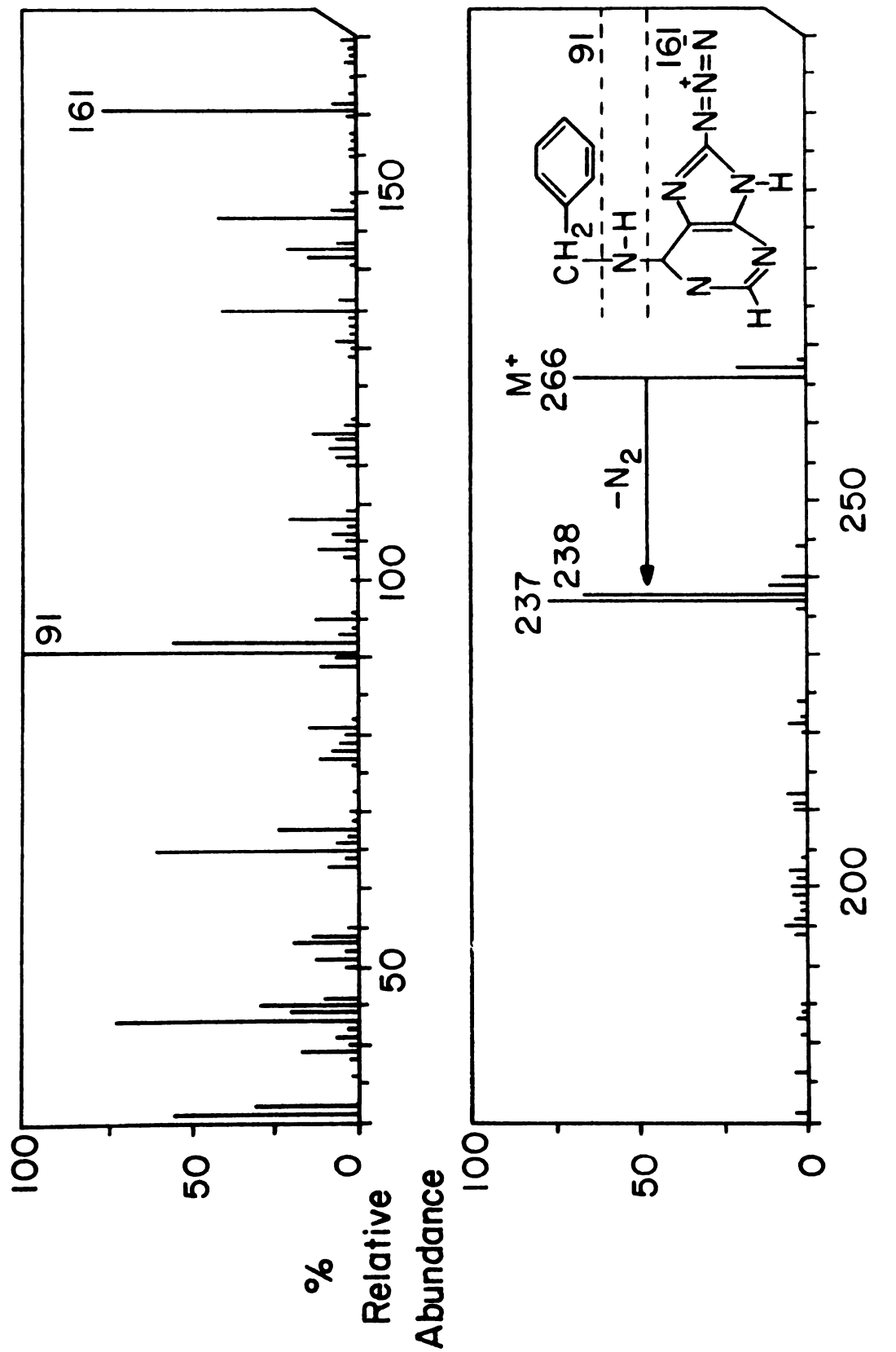

100
50
0
% Relative Abundance
91
161
150
100
50
0
M+
266
237
238
-N2
91
161
CH2
N-H
N=N=N
200
250
m/e

Figure 26

a new suspension culture from a stock of agar-grown callus of the same strain #21 which had been continuously subcultured for just such an evantuality. No other aspect (growth curve, analogue specificity) of the bioassays had changed. It is interesting that with the "newer" liquid cells the higher yield of cells (15 g) could be obtained at high 8-N_3-BA concentrations, while the maximum obtainable yield on BA had been lowered.

Photolysis of 8-N_3-BA

The UV light sources employed in this study were common, hand-held lamps used to detect UV absorbing or fluorescing zones on paper and thin-layer chromatograms: UVS-11 Mineralight for short wavelengths and UVL-21 Blak-Ray for long wavelengths (lamps were obtained from Ultra-Violet Products, San Gabriel, CA, USA). Vessels containing light-sensitive azido derivatives were wrapped in aluminum foil to protect the photoaffinity reagents from exposure to room light. Ultraviolet light intensities were measured with a Kettering Radiant Power Meter (Laboratory Data Control Division, Milton Roy Company, P.O. Box 10235, Riviera Beach, FL 33404).

Spectroscopy

Ultraviolet spectra were recorded on a Carey Model 15 Spectrophotometer and the low resolution mass spectrum was obtained by direct probe using a Varian (Bremen, Germany) Mass Spectrometer Model CH-5 at 70 eV.

Results

Chemical properties and photolysis of 8-N_3-BA

As judged by increased solubility in ethanol and decreased solubility in H_2O, 8-N_3-BA was found to be more lipophilic than BA. Saturation in water was reached at 5×10^{-5} M 8-N_3-BA, as compared to $1\text{-}2 \times 10^{-4}$ M for BA.

The photolysis reaction of 8-N_3-BA could be conveniently monitored by a change in the UV spectrum (Figure 26). This spectral change (in 50% ethanol) was accompanied by a disappearance of the 8-N_3-BA zone at R_f 0.69 in silica gel TLC (Table 10) and the appearance of UV-absorbing compounds at the origin and at R_f 0.08 and also smaller amounts at R_f's 0.04 and 0.89. The photolysis products were not further characterized.

The relative effectiveness of various lamps was determined by measuring the absorbance of unreacted 8-N_3-BA at 305 nm. The photolysis of 8-N_3-BA followed first order kinetics (Figure 28). The short-wavelength UV lamp gave a photolysis rate ($t_{1/2}$ = 1.1 min) twice that of the long-wavelength UV lamp ($t_{1/2}$ = 2.2 min). However, for experiments involving photolysis of 8-N_3-BA in intact tissues, the long-wavelength UV lamp may be more suited. It can also be seen in Figure 28 that a polystyrene petri dish cover reduces the effectiveness of the long-wavelength UV by only 1/3. If it is necessary to maintain sterile conditions during photolysis, the petri dish cover can therefore be kept on and the photolysis time can be increased accordingly.

Biological activity of 8-N_3-BA

In the absence of actinic light, 8-N_3-BA is as active as BA in eliciting bud formation in moss protonemata (Figure 30) and

Figure 27. UV spectrum of 8-N_3-BA before and after various times of photolysis with a long-wavelength UV lamp (900 $\mu W/cm^2$).

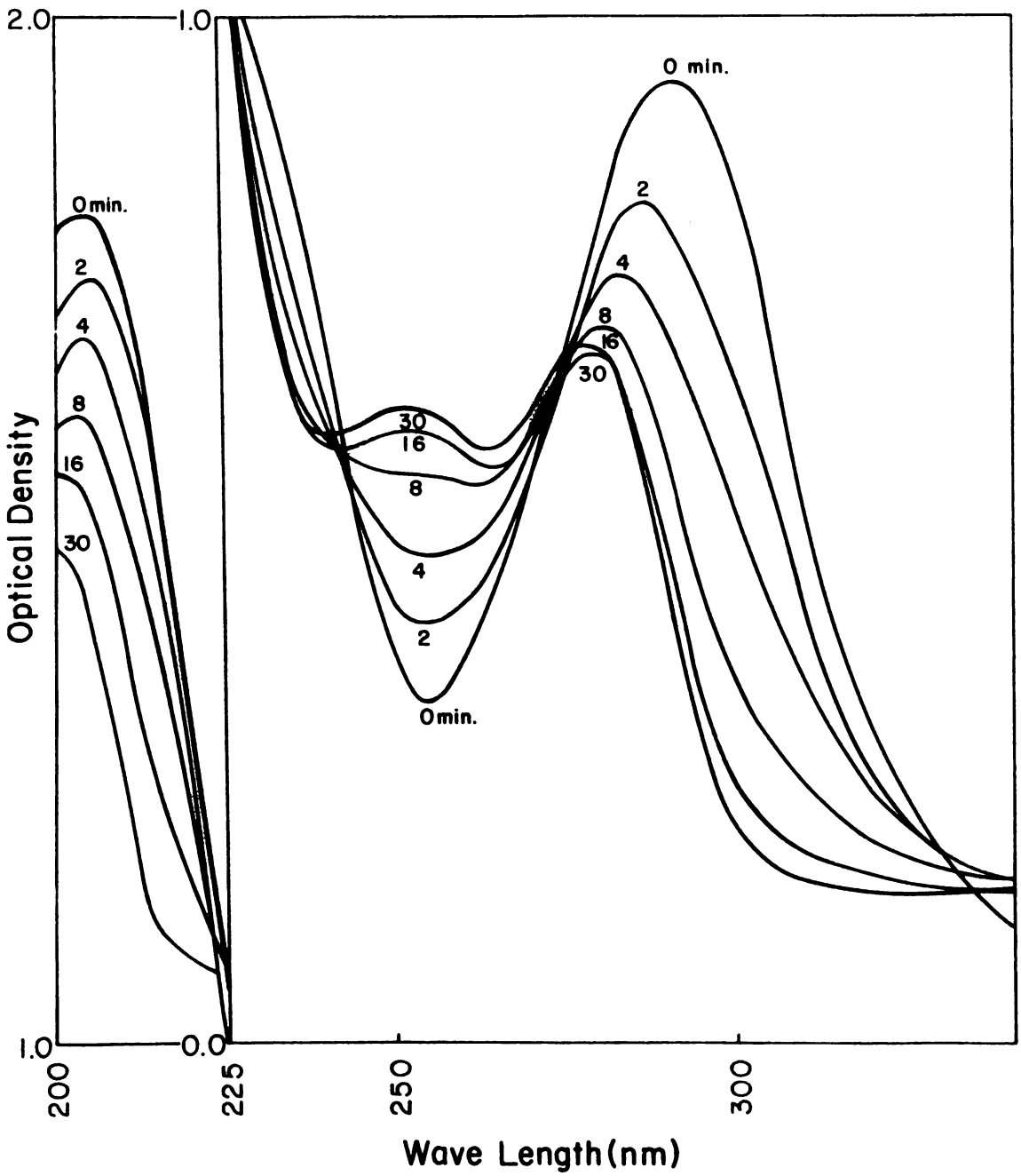

2.0
1.0
1.0
0.0
Optical Density
0min.
2
4
8
16
30
0 min.
2
4
8
16
30
30
16
8
4
2
0min.
200
225
250
300
Wave Length(nm)

Figure 27

Figure 28. Time course of 8-N_3-BA photolysis comparing the effectiveness of long (●) and short (▲) wavelength UV light, with (---) and without (——) a polystyrene petri dish cover as short wavelength filter. The light intensities 0.5 cm from the lamp surface were: short wavelength, 280 $\mu W/cm^2$ (90 $\mu W/cm^2$ with petri dish cover), long wavelength, 900 $\mu W/cm^2$ (630 $\mu W/cm^2$ with petri dish cover).

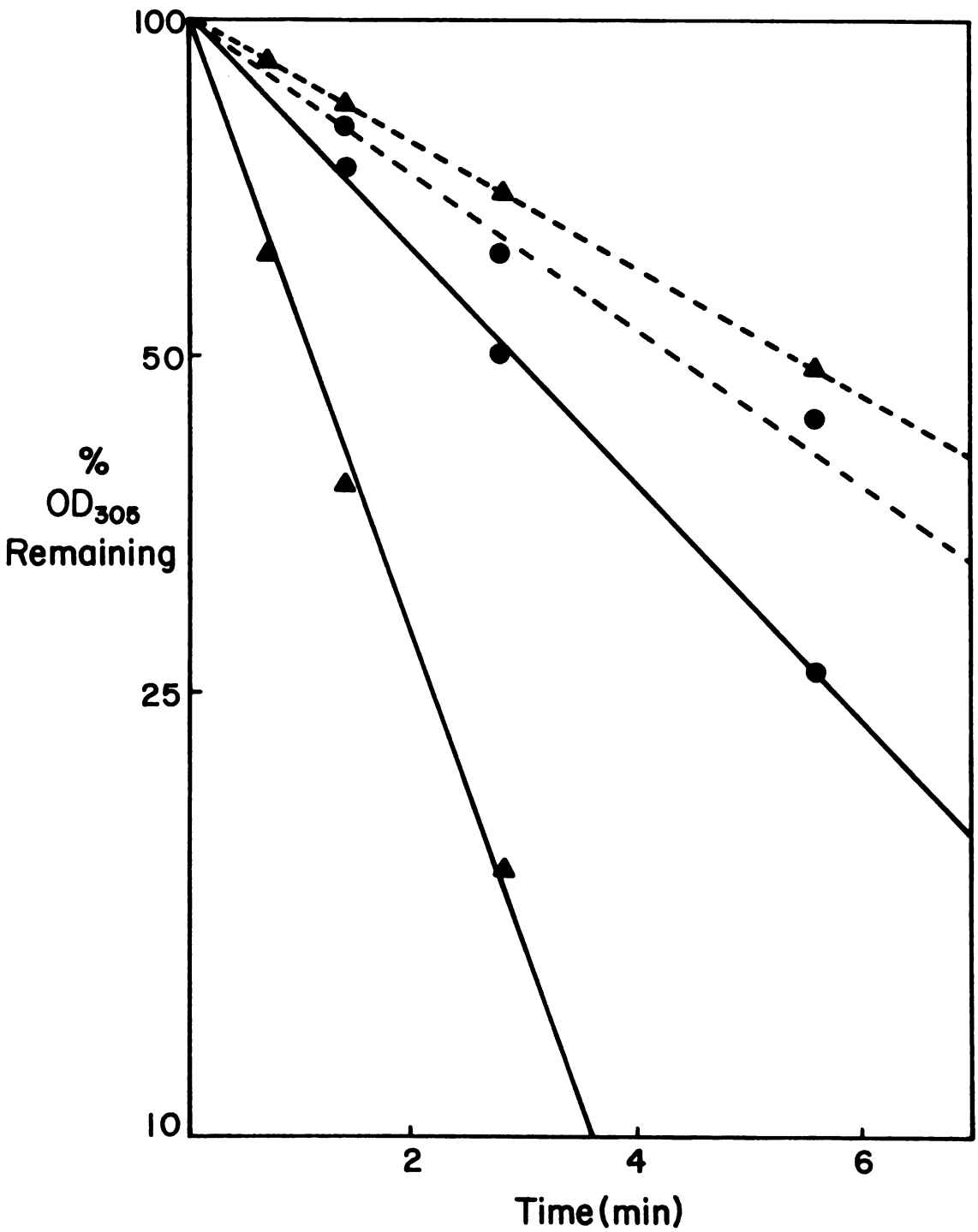


Figure 28

significantly more active than BA in the tobacco cell suspension bioassay (Figure 29). In addition, at concentrations greater than 10^{-6} M, where BA consistently and strongly inhibits growth of tobacco cells, 8-N_3-BA does not. Thus, it appears that 8-N_3-BA has all of the growth-promoting but none of the growth-inhibitory properties of BA in this liquid tobacco cell suspension bioassay. When a high concentration of 8-N_3-BA (2.5×10^{-6} M) was mixed with a supra-optimal concentration of BA (5×10^{-6} M), the yield per flask was 1.3 ± 0.3 g, as compared to 16.5 ± 0.9 g and 16.8 ± 0.9 g when the 8-N_3-BA (2.5×10^{-6} M) was used alone or with 5×10^{-7} M BA, respectively. Thus, cells grown in the presence of high 8-N_3-BA concentrations have not lost their sensitivity to supra-optimal BA concentrations. The inhibitory response at high BA concentrations is less striking and more variable in the moss bioassay.

When a sample of 8-N_3-BA is exposed to short-wavelength UV irradiation (sufficient to obtain $\geq$99% photolysis, as checked by TLC) prior to bioassay, the cytokinin activity is reduced to ca. 1/100 in the moss bioassay (Figure 30) and ca. 1/50 in the tobacco callus bioassay (Figure 29).

Discussion

By a four-step reaction sequence from 8-bromo-adenosine, I was able to synthesize a highly active, light-sensitive cytokinin analogue. Our finding that an 8-azido substitution does not reduce cytokinin activity of BA is in accordance with the known lack of effect of halogen substitution at the 8-position of the purine ring (Dammann and Leonard, 1974) and the chemical similarity of the azido group and the halogens (Boyer and Cantor, 1954). Similarly, the *increased*

Figure 29. Comparison of the cytokinin activity of 8-N_3-BA (●——●), BA (▲——▲) and pre-photolyzed 8-N_3-BA (o---o) in the tobacco cell division bioassay (suspension culture), in the absence of actinic light. The pre-photolyzed 8-N_3-BA sample had been irradiated to ≤1% remaining 8-N_3-BA (as judged by thin layer chromatography) prior to use in the bioassay. Results are expressed as the mean fresh weights ± S.E. of the cells from at least quadruplicate flasks.

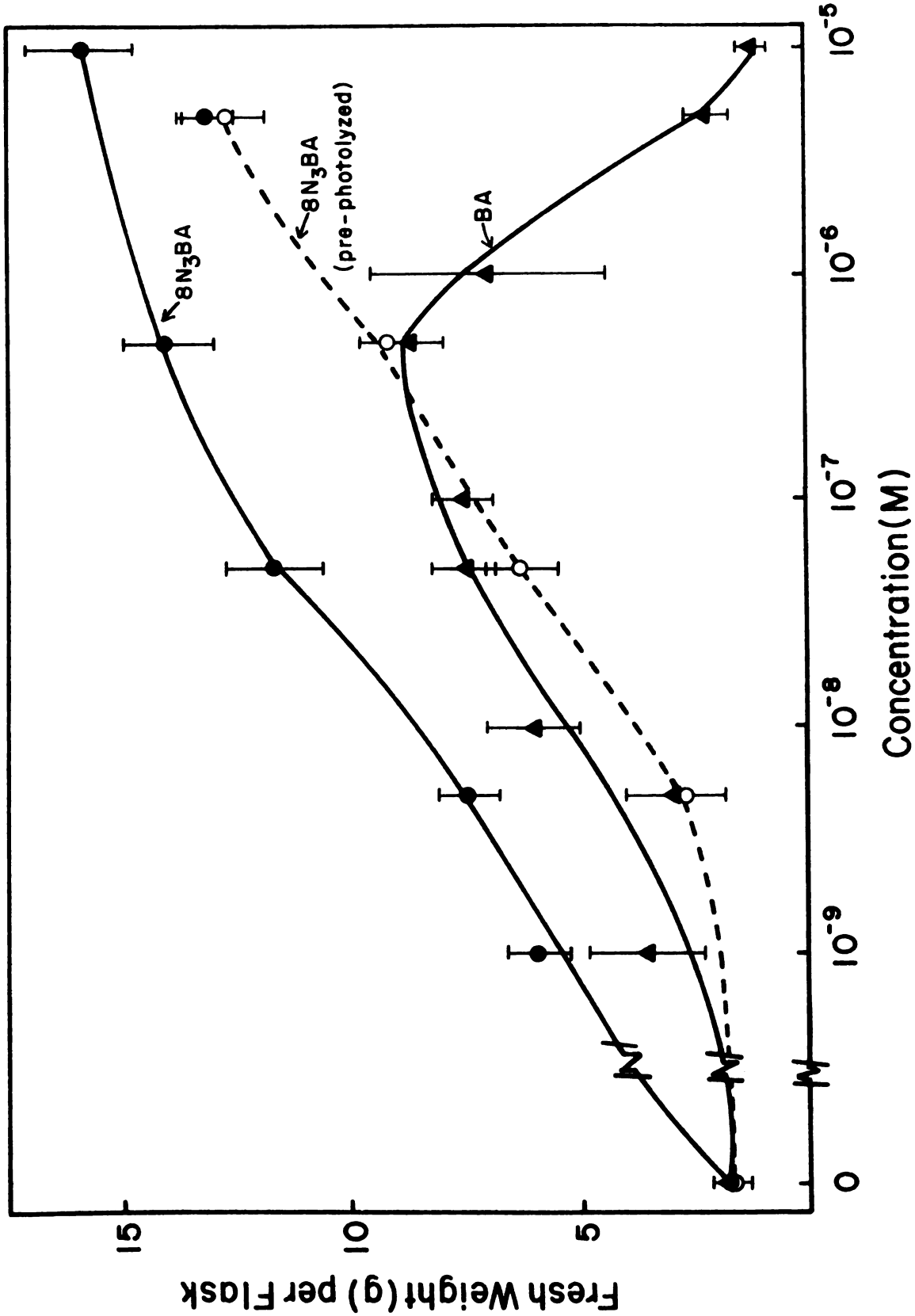

Fresh Weight (g) per Flask
15
10
5
0
M
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
Concentration (M)
$8N_3BA$
$8N_3BA$ (pre-photolyzed)
BA

Figure 29

Figure 30. Comparison of cytokinin activity of 8-N_3-BA (▲——▲), BA (●——●) and pre-photolyzed 8-N_3-BA (o---o) in the moss bud formation bioassay in the absence of actinic light. The same sample of pre-photolyzed 8-N_3-BA was used in this experiment as in the experiment shown in Figure 28. Results are expressed as the mean number of buds per plant ± S.E. of three replicates.

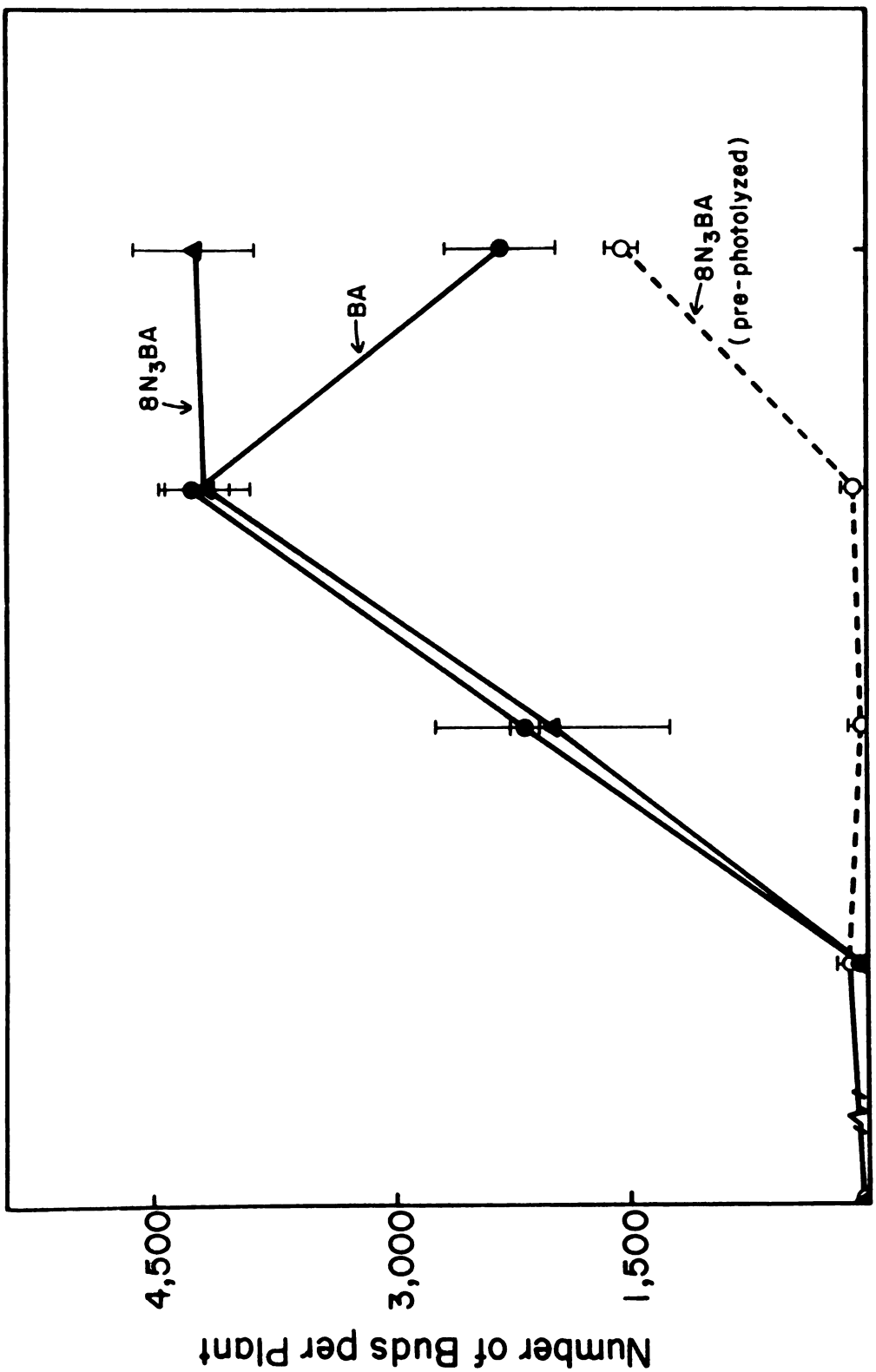

Number of Buds per Plant
4,500
3,000
1,500
0
10^{-8}
10^{-7}
10^{-6}
10^{-5}
Concentration(M)
$8N_3BA$
BA
$8N_3BA$
(pre-photolyzed)

Figure 30

activity of 8-N_3-BA over BA in the liquid tobacco-cell suspension bioassay is consistent with the higher activity of 8-methyl-2iP as compared to 2iP in the tobacco callus bioassay (Dammann and Leonard, 1974). Like a methyl group, the azido group is considered a strong electron donor in an aromatic system (Smith et al., 1962).

One interpretation of the lack of inhibitory activity at high concentrations of 8-N_3-BA as compared to BA (Figure 29) is that there are two receptor sites for BA, one with a low K_d the occupancy of which regulates growth stimulation, and one with a higher K_d which regulates growth inhibition. The lack of inhibition observed with 8-N_3-BA could then be explained by a lack of affinity of 8-N_3-BA for the receptor with the high K_d. The inhibition of growth at high, probably non-physiological concentrations (5×10^{-6} M) of BA may be caused by competition of the cytokinin with substrates of purine-utilizing enzymes. If this is true, there may be more than one "receptor-site" with higher K_d. Alternatively, 8-N_3-BA may be more readily metabolized, and its actual concentration may therefore never reach toxic levels.

The low biological activity we observed with pre-photolyzed 8-N_3-BA in the moss bioassay can probably be accounted for by the presence of remaining, non-photolyzed 8-N_3-BA. Thus, in this system, the products of 8-N_3-BA photolysis are very likely inactive. The results with tobacco, however, are not as clear cut and indicate that the product(s) of photolysis may retain some biological activity in this system.

Since 8-N_3-BA is a fully active cytokinin and has photolytic properties similar to those observed by others with 8-N_3-cAMP and 8-N_3-ATP, its use as a radioactive reagent for a covalent labelling

of cytokinin binding sites is justified. A suitable route to synthesizing tritiated 8-N_3-BA at a high specific activity is available, starting with commercially obtainable 2-^{3}H-adenosine (Amersham Radiochemical Centre). In preliminary experiments, this compound has been successfully brominated to give 2-[^{3}H]-8-bromoadenosine in high yield.

GENERAL DISCUSSION

Contrary to my earliest expectations, not all saturable cytokinin binding is specific binding. This proved to be a much more difficult problem with cytokinins than was reported for analogous auxin binding studies (Hertel et al., 1973). However, in binding studies using radioactive cytokinin of high specific activity (*p*-[^{3}H]-BA, 10 Ci/mmol) and at low concentrations (10^{-9} to 10^{-8} M) a *high*-affinity binding site could be detected in a particulate fraction of tobacco cells. On the basis of its heat lability and its structure-activity properties, which are very similar to those observed in a bioassay using the same cell line, the observed binding site is a likely candidate for the cytokinin receptor.

In a typical experiment, the specifically (*high*-affinity site) bound radioactivity represents only 0.2 to 0.5% of the total radioactivity, similar to the levels reported in the first study on specific auxin binding sites (Hertel et al., 1973). Ordinarily, this might be sufficient for localization of the binding site, but due to the large amounts of non-specific, *low*-affinity binding found with cytokinins, the results are not yet sufficient to resolve a location for specific binding site in the cell.

I observed a distinct difference in the structure-activity requirements in two cytokinin bioassays (moss bud formation and tobacco cell division) which was interpreted on the basis of differences in binding sites of the respective receptors. Though other

interpretations are possible, this one can be directly tested by binding experiments using moss homogenates in a fashion similar to those which have been carried out with tobacco. However, it should be noted that a 75-90% reduction in the number of high-affinity binding sites observed in the particulate fraction of tobacco cells (see Figure 22) would probably have made their detection impossible because of the high background of *low*-affinity, non-specific binding. This problem may be insurmountable in the moss system since only a fraction of the cells in any one protonema are responsive to cytokinin.

For a more detailed characterization of the specific, *high*-affinity cytokinin binding site in tobacco cells, I synthesized a biologically active cytokinin photoaffinity reagent, 8-N_3-BA. This approach is expected to reduce the problems arising from non-specific cytokinin binding because light-generated covalent binding via nitrene insertion to a specific, higher-affinity binding sites has been reported to have a higher efficiency than that to lower affinity, non-specific binding sites (see Hixson and Hixson, 1973, for discussion of this point). In addition, this technique is amenable to enrichment of the specific binding signal by the use of gel electrophoresis under denaturing conditions (SDS), once the covalent bond is generated. Thus, if a gel is divided into 100 slices, each containing one theoretical non-specific binding site, the signal-to-noise ratio for detecting specific binding to a protein in one slice is effectively increased by a factor of 100.

Ultimately, proof must be obtained that a specific binding site is or is not involved in the physiological response. As was pointed out by Kende and Gardner (1976), this can only be achieved by two

approaches: (1) examining tissues with a single point genetic lesion affecting the receptor, and (2) characterization of *in vitro* biochemical reactions which are also observed in the early *in vivo* response of the tissue to the hormone.

REFERENCES

REFERENCES

Armstrong, D. J., Burrows, W. J., Evans, P. K., Skoog, F. S.: Isolation of cytokinins from tRNA. Biochem. Biophys. Res. Commun. 37, 451-456 (1969).

Batt, S., Wilkins, M., Venis, M. A.: Auxin binding to corn coleoptile membranes: kinetics and specificity. Planta (Berl.) 130, 7-13 (1976).

Batt, S., Venis, M. A.: Separation and localization of two classes of auxin binding sites in corn coleoptile membranes. Planta (Berl.) 130, 15-21 (1976).

Berridge, M. V., Ralph, R. K., Letham, D. S.: The binding of kinetin to plant ribosomes. Biochem. J. 119, 75-84 (1970).

Berridge, M. V., Ralph, R. K., Letham, D. S.: On the significance of cytokinin binding to plant ribosomes. In: *Plant Growth Substances*, 1970, pp. 248-255. Ed.: Carr, D. J., Berlin: Springer-Verlag 1972.

Bezemer-Sybrandy, S. M., Veldstra, H.: Investigations on cytokinins. IV. The metabolism of BA in *Lemna minor*. Physiol. Plant 25, 1-7 (1971).

Boyer, J. H., Cantor, F. C.: Alkyl and aryl azides. Chem. Rev. 56, 1-57 (1954).

Brandes, H., Kende, H.: Studies on cytokinin-controlled bud formation in moss protonemata. Plant Physiol. 43, 827-837 (1968).

Cleland, R. E.: Auxin-induced hydrogen ion excretion: correlation with growth, and control by external pH and water stress. Planta (Berl.) 127, 233-242 (1975).

Cuatrecasas, P., Tell, C. P. E., Sica, V., Parikh, I., Chang, K.: Noradrenalin binding and the search for catecholamine receptors. Nature 247, 92-97 (1974).

Cuatrecasas, P., Hollenberg, M. D.: Binding of insulin and other hormones to non-receptor materials: saturability, specificity and apparent "negative cooperativity." Biochem. Biophys. Res. Commun. 62, 31-41 (1975a).

Cuatrecasas, P., Hollenberg, M. D., Chang, K., Bennett, V.: Hormone receptor complexes and their modulation of membrane function. In: *Recent Progress in Hormone Research*, Vol. 31, pp. 37-94. New York: Academic Press 1975b.

Cuatrecasas, P., Hollenberg, M. D.: Membrane receptors and hormone action. Adv. Protein Chem. 30, 251-451 (1976).

Dammann, L. G., Leonard, N. E.: Cytokinins: synthesis of 2-, 8-, and 2,8-substituted 6-(3-methyl-2-butenyl)aminopurines and their relative activities in promoting cell growth. Phytochem. 13, 329-336 (1974).

Davies, E., Larkins, B. A.: Polyribosomes from peas. V. An attempt to characterize the total free and membrane-bound polysomal population. Plant Physiol. 55, 749-756 (1975).

Dohrmann, U.: Charackterisierung der *in-vitro*-bindung des pflanzenhormons auxin bei koleoptilen von *Zea mays* L. Inaugural-Dissertation Albert-Ludwigs-Universitat, Freiburg i. Br. 85pp (1975).

Dollstadt, R., Hirschberg, K., Winkler, E., Hubner, G.: Bindung von indolylessigsaure und phenoxyessigsaure an fraktionen aus epikotylen und wurzeln von *Pisum sativum* L. Planta (Berl.) 130, 105-111 (1976).

Dyson, W. H., Hall, R. H., Hong, C. I., Dutta, S. P., Cheda, G. B.: Cytokinin activity of a group of ureidopurine derivatives related to the tRNA component, N-(purin-6-ylcarbamoyl)threonine. Can. J. Bot. 50, 237-243 (1972).

Elion, G. B., Burgi, E., Hitchings, G. H.: Studies on condensed pyrimidine systems. IX. The synthesis of some 6-substituted purines. J. Am. Chem. Soc. 74, 411-414 (1952).

Elliott, D. C., Murray, A. W.: A quantitative limit for cytokinin incorporation into tRNA by soya-bean callus tissue. Biochem. J. 130, 1157-1160 (1972).

Evans, E. A., Sheppard, H. C., Turner, J. C.: Validity of tritium tracers. Stability of tritium atoms in purines, pyrimidines, nucleosides and nucleotides. J. Labelled Cmpds. 6, 76-87 (1970).

Farrow, J. T., Vunakis, H. V.: Binding of d-lysergic acid diethylamide to subcellular fractions from rat brain. Nature 237, 164-166 (1972).

Fleck, A., Munro, H. N.: The precision of UV absorption measurements in the Schmidt-Thannhauser procedure for nucleic acid estimation. Biochim. Biophys. Acta 55, 571-583 (1962).

Fox, J. E., Erion, J. L.: A cytokinin binding protein from higher plant ribosomes. Biochem. Biophys. Res. Commun. 64, 694-700 (1975).

Fox, J. E., Sood, C. K., McChesney, J. D.: Effect of substituents at the 9-position on cytokinin activity. Phytochem. 12, 1531-1533 (1973).

Goldstein, A.: Opiate receptors, a minireview. Life Sci. 14, 615-623 (1974).

Guthrow, C. E., Rasmussen, H., Brunswick, D. J., Cooperman, B. S.: Specific photo-affinity labeling of the cAMP receptor in intact ghosts from human erythrocytes. Proc. Nat. Acad. Sci. (Wash.) 70, 3344-3346 (1973).

Haley, B. E.: Photo-affinity labeling of cAMP binding sites of human red cell membranes. Biochemistry 14, 3852-3857 (1975).

Haley, B. E., Hoffman, J. F.: Interactions of a photo-affinity ATP analogue with cation stimulated ATPases of human red cell membranes. Proc. Nat. Acad. Sci. (Wash.) 71, 3367-3371 (1974).

Hall, Z. W.: Release of neurotransmitters and their interactions with receptors. Ann. Rev. Biochem. 41, 925-952 (1972).

Hanstein, W., Hatefi, Y.: Characterization and localization of mitochondrial uncoupler binding sites with an uncoupler capable of photo-affinity labeling. J. Biol. Chem. 249, 1356-1362 (1974).

Hecht, S. M., Frye, R. B., Werner, D., Hawrelack, S. D., Skoog, F., Schmitz, R. Y.: On the "activation" of cytokinins. J. Biol. Chem. 250, 7343-7351 (1975).

Hertel, R., St. Thomson, K., Russo, V. E. A.: *In-vitro* auxin binding to particulate cell fractions from corn coleoptiles. Planta (Berl.) 7, 325-340 (1972).

Hertel, R.: Auxin transport and *in vitro* auxin binding. In: *Membrane Transport in Plants*, pp. 457-461. Ed.: Zimmerman, M., Dainty, J., Heidelberg: Springer 1974.

Hixson, S. S., Hixson, S. H.: Photochemical labeling of yeast alcohol dehydrogenase with an azide analog of NAD^+. Photochem. Photobiol. 18, 135-138 (1973).

Hodges, T. K., Leonard, R. T.: Purification of an ion stimulated ATPase from plant roots. In: *Methods in Enzymology*, Vol. 32, pp. 392-406. Ed.: Colowick, S. P., Kaplan, N. O., New York: Academic Press 1973.

Holmes, R. E., Robins, R. K.: Purine nucleosides. VII. Direct bromination of adenosine, deoxyadenosine, guanosine, and related purine nucleosides. J. Amer. Chem. Soc. 86, 1242-1245 (1964).

Holmes, R. E., Robins, R. K.: Purine nucleosides. IX. The synthesis of 9-β-D-ribofuranosyl uric acid and other related 8-substituted purine ribonucleosides. J. Amer. Chem. Soc. 87, 1772-1776 (1965).

Jensen, E. V., DeSombre, E. R.: Mechanism of action of the female sex hormones. Ann. Rev. Biochem. 41, 203-230 (1972).

Jouanneau, J. P., Réaud-Lenoël, C.: Croissance et synthese des proteines de suspensions cellulaire de Tabac sensible a la kinetine. Physiol. Plant 20, 834-850 (1967).

Keates, R. A. B., Trewavas, A. J.: Protein kinase activity associated with isolated ribosomes from peas and *Lemna*. Plant Physiol. 54, 95-99 (1974).

Kasamo, K., Yamaki, T.: *In vitro* binding of IAA to plasma membrane-rich fractions containing Mg^{+2}-activated ATPase from mung bean hypocotyls. Plant Cell Phys. 17, 149-164 (1976).

Kende, H.: The cytokinins. Int. Rev. Cytology 31, 301-337 (1971).

Kende, H., Tavares, J.: On the significance of cytokinin incorporation into RNA. Plant Physiol. 43, 1244-1248 (1968).

Kende, H., Hahn, H., Kays, S. E.: Enhancement of nitrate reductase by benzyladenine in *Agrostemma githago*. Plant Physiol. 48, 702-706 (1971).

Kende, H., Gardner, G.: Hormone binding in plants. Ann. Rev. Plant Physiol. 27, 267-290 (1976).

Knowles, J. R.: Photogenerated reagents for biological receptor-site labeling. Accounts Chem. Res. 5, 155-160 (1972).

Kuraishi, S.: Effect of kinetin analogs on leaf growth. Sci. Papers College Gen. Educ., Univ. Tokyo 9, 67-104 (1959).

Leonard, N. J., Fuji, T.: The synthesis of compounds possessing kinetin activity. The use of a blocking group at the 9-position of adenine for the synthesis of 1-substituted adenines. Proc. Nat. Acad. Sci. (Wash.) 51, 73-75 (1964).

Leonard, N. J.: Chemistry of the cytokinins. In: *The Chemistry and Biochemistry of Plant Hormones*, Vol. 7, pp. 21-56. Ed.: Runeckles, V. C., Sondheimer, E., Walton, D. C., New York: Academic Press 1974.

Letham, D. S.: Regulators of cell division in plant tissues. V. A comparison of the activities of zeatin and other cytokinins in five bioassays. Planta (Berl.) 74, 228-242 (1967).

Letham, D. S., Young, H.: The synthesis of radioisotopically labelled zeatin. Phytochem. 10, 2077-2081 (1971).

Linsmaier, E. M., Skoog, F.: Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant 18, 100-127 (1965).

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J.: Protein measurement with the Folin-Phenol reagent. J. Biol. Chem. 193, 265-275 (1951).

Maassen, J. A., Moller, W.: Identification by photo-affinity labeling of the proteins in *Escherichia coli* ribosomes involved in elongation factor G-dependent GDP binding. Proc. Nat. Acad. Sci. (Wash.) 71, 1277-1280 (1974).

Malkinson, A. M., Krueger, B. K., Rudolph, S. A., Casnellie, J. E., Haley, B. E., Greengard, P.: Widespread occurrence of a specific protein in vertebrate tissues and regulation by cAMP of its endogenous phosphorylation and dephosphorylation. Metabolism 24, 331-341 (1975).

Miflin, B. J., Beevers, H.: Isolation of intact plastids from a range of plant tissues. Plant Physiol. 53, 870-874 (1974).

Miller, C. O., Skoog, F., Von Saltza, M. H., Strong, F. M.: Kinetin, a cell division factor from deoxyribonucleic acid. J. Amer. Chem. Soc. 77, 1392 (1955).

Muneyama, K., Bauer, R. J., Shuman, D. A., Robins, R. K., Simon, L. N.: Chemical synthesis and biological activity of 8-substituted cAMP derivatives. Biochemistry 10, 2390-2395 (1971).

Nathanson, J. A., Greengard, P.: Serotonin-sensitive adenylate cyclase in neural tissue and its similarity to the serotonin receptor: a possible site of action of LSD. Proc. Nat. Acad. Sci. (Wash.) 71, 797-801 (1974).

Okumura, F. S., Kotani, Y., Ariga, T., Masumura, M., Kuraishi, S.: Syntheses of kinetin-analogues. Bull. Chem. Soc. Japan 32, 883-887 (1959).

Oostrom, H., Van Loopik-Detmers, M. A., Libbenga, K. R.: A high affinity receptor for indoleacetic acid in cultured tobacco pith explants. FEBS Letters 59, 194-197 (1975).

Pomerantz, A. H., Rudolph, S. A., Haley, B. S., Greengard, P.: Photo-affinity labeling of protein kinase from bovine brain with 8-azido-cAMP. Biochemistry 14, 3858-3862 (1975).

Puca, G. A., Nola, E., Sica, V., Bresciani, F.: Estrogen-binding proteins of calf uterus. Partial purification and preliminary characterization of two cytoplasmic proteins. Biochemistry 10, 3769-3779 (1971).

Ralph, R. K., McCombs, P. J. A., Tener, G., Wojcik, S. J.: Evidence for modification of protein phosphorylation by cytokinins. Biochem. J. 130, 901-911 (1972).

Ralph, R. K., Bullivant, S., Wojcik, S. J.: Effects of kinetin on phosphorylation of leaf membrane proteins. Biochim. Biophys. Acta 421, 319-327 (1976).

Riggs, A. D., Bourgeois, S.: On the assay, isolation and characterization of the *lac* repressor. J. Mol. Biol. 34, 361-364 (1968).

Rosenthal, H. E.: A graphic method for the determination and presentation of binding parameters in a complex system. Anal. Biochem. 20, 525-532 (1967).

Scatchard, G.: The attractions of proteins for small molecules and ions. Ann. N.Y. Acad. Sci. 51, 660-672 (1949).

Sharma, S. K., Nirenberg, M., Klee, W. A.: Morphine receptors as regulators of adenylate cyclase activity. Proc. Nat. Acad. Sci. (Wash.) 72, 590-594 (1975).

Shelton, K. R., Clark, Jr., J. M.: A proton exchange between purines and water and its application to biochemistry. Biochemistry 6, 2735-2739 (1967).

Smith, P. A. S., Hall, J. H., Kan, R. O.: The electronic character of the azido group attached to benzene rings. J. Amer. Chem. Soc. 73, 485-489 (1962).

Soriano-Garcia, M., Parthasarthy, R.: Structure-activity relationship of cytokinins: crystal structure and conformation of kinetin. Biochem. Biophys. Res. Commun. 64, 1062-1068 (1975).

Spudich, J. L., Koshland, D. E., Jr.: Quantitation of the sensory response in bacterial chemotaxis. Proc. Nat. Acad. Sci. (Wash.) 72, 710-713 (1975).

Sussman, M. R., Firn, R. D.: The synthesis of a radioactive cytokinin with high specific activity. Phytochem. 15, 153-155 (1976).

Takegami, T., Yoshida, K.: Isolation and purification of cytokinin binding protein from tobacco leaves by affinity chromatography. Biochem. Biophys. Res. Commun. 67, 782-789 (1975).

Tandeau de Marsac, N., Jouanneau, J.: Variation de l'exigence en cytokinine de lignees clonales ce cellules de Tabac. Physiol. Veg. 10, 369-380 (1972).

Thomson, K. S.: The binding of 1-N-naphthylphthalamic (NPA), an inhibitor of auxin transport, to particulate fractions of corn coleoptiles. In: *Hormonal Regulation in Plant Growth and Development*. Proc. Adv. Study Inst. Izmir 1971, pp. 83-88. Ed.: Kaldeway, H., Vardar, Y., Weinheim: Verlag-Chemie 1972.

Thomson, K., Hertel, R., Muller, S. Tavares, J. E.: NPA and TIBA *in vitro* binding to particulate cell fractions and action on auxin transport in corn coleoptiles. Planta (Berl.) 109, 337-352 (1973).

Thomson, K. S., Leopold, A. C.: *In vitro* binding of morphactins and 1-N-naphthylphthalamic acid in corn coleoptiles and their effect on auxin transport. Planta (Berl.) 115, 259-270 (1974).

Walker, G. C., Leonard, N. J., Armstrong, D. J., Murai, N., Skoog, F.: The mode of incorporation of BA into tobacco callus tRNA; a double labeling determination. Plant Physiol. 54, 737-743 (1974).

Whitaker, B. D., Kende, H.: Bud formation in *Funaria hygrometic*: A comparison of the activities of three cytokinins with their ribosides. Planta (Berl.) 121, 93-96 (1974).

Whitfield, P.: Detection of purines, pyrimidines, nucleosides and nucleotides. In: *Data for Biochemical Research*, p. 565. Ed.: Dawson, R. M. C., Elliott, W. H., Jones, K. M., New York: Oxford University Press 1969.

Zubay, G., Schwartz, D., Beckwith, J.: Mechanism of activation of catabolite-sensitive genes: a positive control system. Proc. Nat. Acad. Sci. (Wash.) 66, 104-110 (1970).