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PARTIAL CHARACTERIZATION AND PURIFICATION OF A GIBBERELLIN BINDING PROTEIN FRACTION FROM CUCUMBER SEEDLINGS

by

Nasser Yalpani

B.Sc., Simon Fraser University, 1977

M.P.M., Simon Fraser University, 1981

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Partial characterization and purification of a gibberellin binding protein fraction from cucumber seedlings

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ABSTRACT

A DEAE filter paper assay was used for the partial purification of gibberellin binding protein (GABP) containing extracts from cucumber seedlings. The procedures used for this *in vitro* assay were refined to allow reliable detection of GABPs with characteristics expected of GA receptors.

The GA binding properties of a cytosol fraction from hypocotyls was examined using [^{3}H]GA_4 and over 20 GAs, GA derivatives and other growth substances. The results demonstrate structural specificity of the binding protein for γ-lactonic C-19 GAs with a 3 β-hydroxyl and a C-6 carboxyl group. Additional hydroxylations of the A, C, or D ring of the *ent*-gibberellane skeleton or methylation of the C-6 carboxyl impede or abolish binding affinity. These *in vitro* results are generally supported by *in vivo* bioassay data. However, GA_9 and GA_{36}, both considered to be precursors of the presumably active GA_4 in cucumber, have low affinity for the binding protein. With these data inferences about the active site of the putative GA_4 receptor in cucumber were made possible.

Partial purification of the GABP fraction was achieved by $(NH_4)_2SO_4$ fractionation and DEAE cellulose chromatography. Further purification could be achieved using a hydroxylapatite or Blue dextran-agarose column, or by pH fractionation. FPLC-chromatofocusing and -anionexchange chromatography did not result in enhanced specific binding.

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PART A

Introduction

Since their structural identification in the 1950's, gibberellins (GAs) have been recognized to be present throughout most of the plant kingdom and to affect a wide range of physiological processes in plant development (see Figure 6.4 in Matthysse and Scott 1984). The most dramatic effect involves the promotion of stem growth in genetic or physiological dwarf plants. GAs also induce germination of many photodormant seeds, substrate mobilization in cereal grains, as well as leaf elongation and parthenocarpic fruit set. They also control flowering and sex expression in some gymno- and angiosperms (Brian 1966, Jones 1973, Pharis 1985).

Numerous investigators have examined the physiological responses to external GA applications or attempted to identify the effect of changes in endogenous GA levels. However, the sequence of events involved in linking GAs with their developmental effects remains largely unresolved. Any hypothesis on the mechanism of GA action has to account for three important facts. 1. GAs can affect transcription of genes and translation of gene products within several hours of application (Deikman and Jones 1986, Hammerton and Ho 1986, Higgins et al. 1976, Jacobsen 1983, Mundy et al. 1985, Mundy et al. 1986, Muthukrishnan et al. 1983a). 2. These effects are observed only in response to a few of the more than 70 GAs of plant and fungal origin (Crozier and Durley 1983). 3. The degree of activity and the type of biological effect are also determined by the stage of differentiation and development of the plant tissue (Katsumi

and Kazama 1978).

The specificity of the relation between biological activity and GA structure as well as the diversity of processes regulated by GAs suggest the presence of GA recognition molecules, receptors, that act as key mediators in GA action in plants. The structural complexity required of such receptors suggests that they are proteins. A number of studies have been undertaken to demonstrate the presence of and ascribe a functional role to such receptor molecules. These studies generally utilized techniques similar to those used by endocrinologists studying the mechanism of action of animal hormones who, in the last 2 decades, have clearly shown that receptor proteins play a key role in mediating the effect of steroid and peptide hormones on gene expression and on metabolism (Greene and Press 1986, Scheidereit et al. 1986, Schrader et al. 1981). Based upon evidence from animal hormone receptors, it is envisaged that plant hormone receptors are located on or in responsive cells. Upon interaction with a growth regulator they become activated, or combine with it, and then, either alone or in a complex with the ligand, exercise metabolic control.

Reviews by Kende and Gardner (1976), Rubery (1981), Stoddart (1982), Stoddart and Venis (1980) and Venis (1977), indicated that there is little experimental support for the existence of candidate GA-receptor proteins. However, in light of more recent research a reexamination of the published reports is appropriate and will, therefore, be the focus of a literature review. In

this review, evidence in support of the existence of GA receptors is presented. On this basis experiments were performed to assay, characterize and purify such proteins from extracts of cucumber seedlings.

PART B

Literature Review

I. Introduction

Within the last two decades a number of researchers have attempted to identify and isolate the primary site of GA action within plants. However, compared to the understanding gained about the mechanism of action of auxins (Jacobsen and Hajek 1987, Libbenga et al. 1987, Loebler et al. 1987, Venis 1987), progress in GA receptor research has been slow. Several factors may account for this. GA receptors are expected to be present at low concentrations (picomolar) in cells. Thus purification of these proteins is a formidable task. Furthermore, in order to be able to detect such a small number of molecules, radioactive GAs of high purity and specific activity are required. A reliable source of such labeled GAs did not exist until recently.

In this part of the thesis a review of the characteristics expected of GA receptors and the methods used for their measurement will be followed by an examination of the available literature on GA-binding cell constituents. A background discussion of a model system of GA action, the cereal aleurone, is provided in Appendix 1.

II. Expected Characteristics of GA Receptors

A protein has to fulfill a number of criteria in order to qualify as a receptor. These criteria have been discussed in detail by several authors (Birnbaumer et al. 1974, Buhler et al. 1976, Clark and Peck 1980, Kende and Gardner 1976, Smith and Sestill 1980, Stoddart and Venis 1980) and will, therefore, only be briefly summarized.

1. Finite binding capacity

The in vivo response to external GA application is saturable (eg., Brian et al. 1964). However, saturation of uptake is not always observed (see Keith et al. 1980). Transport, metabolism, and/or inactivation by compartmentation by eg., vacuoles (Garcia-Martinez et al. 1981) change the hormone concentration at the target site. However, since receptor numbers are limited, saturable binding of hormone to receptor molecules should be measurable once the impact of these processes has been reduced. Saturation is usually demonstrated by exposing the receptor to a range of concentrations of radioactively labeled hormone and subsequently determining the amount of hormone bound.

2. High affinity and reversibility

GA concentrations in plant tissues are usually very low (pmol or less than 100 ng g^{-1} fresh weight) (Gaskin et al. 1985, Oden et al. 1987). GA receptors are, therefore, expected to have a high affinity for biologically active GAs. The equilibrium dissociation constant (K_d) of GA receptors is likely to be of the same order of magnitude as the tissue GA concentration (10^{-7} to 10^{-9} M) (see Clark and Peck 1980, Venis 1985).

Physiological responsiveness to changes in GA concentrations implies reversible and hence noncovalent binding of hormone by the receptor (Venis 1985).

3. Hormone specificity and receptor lability

GA receptors are expected to display high specificity for GAs, thus enabling them to respond to a hormonal signal without interference from other substances. Among the GAs, in vitro binding affinity should roughly correlate with the relative biological activity of that molecule. However, correlation may be poor if factors other than receptor affinity, such as transport, permeability, and perhaps more importantly metabolic conversions affect the in vivo activity of a substance (eg., Nash et al. 1978).

On the basis of in vivo bioassays and studies of GA metabolism (eg., Brian et al. 1967, Crozier 1981, Serebryakov et

al. 1984) the following structural features appear to be important for receptor binding of GAs or GA derivatives: a complete gibbane ring system with a γ-lactone ring, a β-carboxyl group at C-6 and a 3 β-hydroxyl group at C-3. Models of the GA binding sites of the pea and cucumber receptors were proposed by Serebryakov et al. (1984) (Fig. B1). The specificity of GA binding sites can be analyzed by comparing the concentrations of different ligands that competitively inhibit 50% of the high affinity binding of a biologically active, radiolabeled GA.

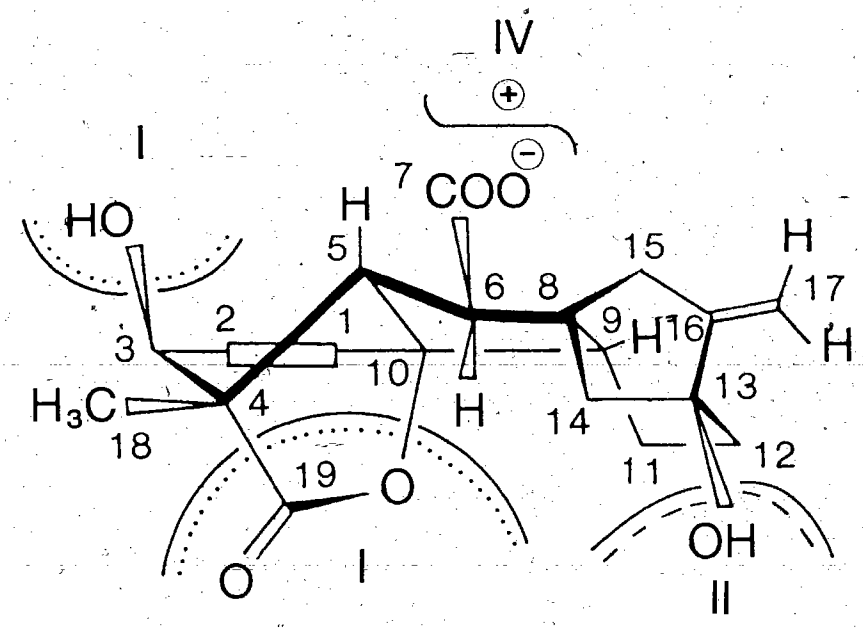
The observed structural specificity implies that the GA receptor is a protein. Specific binding is, therefore, expected to be diminished by heat and protease treatment.

4. Tissue specificity

The in vivo effect of GAs on plants is influenced by the type and physiological state of the tissue to which the hormone is applied. Thus in cucumber hypocotyls, GA_3 more effectively induces elongation in younger than in older tissues (Katsumi and Kazama 1978). In 1 day old lettuce seedlings, maximum hypocotyl growth occurs when GA_3 is applied after an initial 4 or 8 h of seedling growth in water. After 24 h little growth promotion is observed (Sawhney and Srivastava 1974).

Such differences in tissue sensitivity to GA could result from differences in gene availability and/or receptor numbers. There is also the possibility that receptor types differ between

Figure B1. Hypothetical sites of binding between a C-19 GA and a specific receptor in the case of optimal spatial correspondence: a) GA_3 and the dwarf pea receptor; b) GA_7 and the cucumber receptor.
The following sites may be conceived on the basis of bioassay data: (I) Site of obligatory binding - a good fit here is necessary for high activity; (II) sites of ancillary binding - a good fit here increases the activity by some degree; (III) specific site of obligatory hydrophobic interaction; (IV) site of electrostatic interaction between the ionized carboxyl group on the hormone and a positively charged group on the receptor surface. The binding at the alcoholic hydroxyl groups and at the lactone bridge may be assured either by hydrogen bonds or by transient formation of an ester linkage. (Copied from Serebryakov et al. 1984, with permission from Pergamon Press). The carbon numbers are shown in a).


IV
I
HO
H
7 COO
5
15
H
17
6 8
2
1
9
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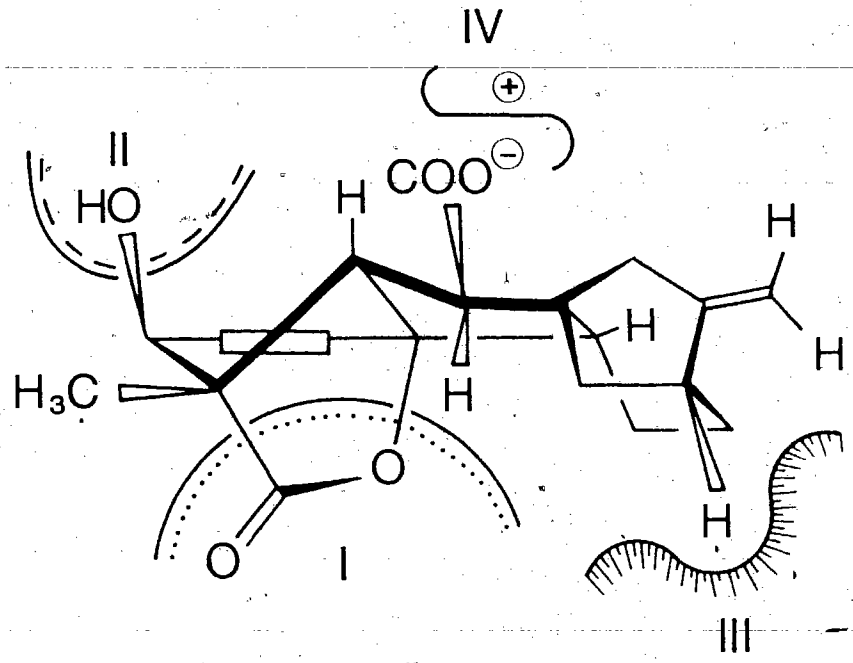
16
H
3
10
H3C
4
H
14
13
18
19
O
11
12
O
I
OH
II

(a)


IV
II
HO
H
COO
H
H
H
H3C
H
O
H
O
I
III

(b)

tissues. Thus Michniewicz and Lang (1962) reported that in Myosotis GA_3 induces only stem growth while GA_7 promotes stem growth and flowering. Brian et al. (1962) reported that in Grand Rapids lettuce the order of effectiveness in ability to promote seed germination by $GA_7 > GA_3 > GA_4$, but ability to promote stem elongation by $GA_4 = GA_7 > GA_3$. It should be noted though that Crozier et al. (1970) and Keith et al. (1979) observed lettuce hypocotyl elongation by $GA_7 > GA_3 > GA_4$. These experiments should be reexamined.

5. Correlation with biological response

The demonstration that hormone-receptor binding is biologically relevant and leads to a hormonal response is difficult to establish. Comparison of receptor deficient or enriched single gene mutants with normal plants can help to determine its biological role. GA receptor mutants have not yet been identified. Ho et al. (1980) screened sodium-azide mutagenized barley for altered sensitivity to GA_3. Various GA_3 insensitive or supersensitive mutants were identified. Some of these lacked both the GA_3-enhanced production of α-amylase and release of phosphatase. The rate of uptake of [^{14}C]GA_3 in these mutants was the same as in the wild-type. The authors suggested that the mutation affected a regulatory step that controlled both enzymes. Other candidate mutants are the GA insensitive maize d_8 (Katsumi et al. 1984, Phinney 1956), wheat D6899 (Ho et al. 1981) and pea lk mutants (Reid and Potts 1986).

III. Receptor Assay

The measurement of receptor-GA complex formation is essential for the quantitation and characterization of a putative receptor. Such analysis usually requires the use of radiolabeled hormone of high purity and specific activity because receptor numbers are expected to be low.

A number of techniques have been developed to separate ligand molecules bound to the receptor from free or unbound ligand molecules (see reviews by Birnbaumer 1980, Venis 1985). Particulate receptor-hormone complexes are readily separated from free ligand by filtration or centrifugation techniques. This is especially suitable for membrane-bound receptors. Separation of ligand complexed with soluble binding sites from free hormone can be achieved by gel filtration (Schrader 1975) or by charcoal adsorption of free ligand (Santi et al. 1973). Protein-hormone complexes may adsorb to ion exchange celluloses (Santi et al. 1973). They may also be precipitated with ammonium sulfate or trichloroacetic acid and then washed free of unbound hormone (Schrader 1975, Smith and Sestill 1980). Receptor-type proteins have also been identified and quantified using specific antibodies (eg., Dicker et al. 1984), affinity crosslinking reagents (Jones et al. 1984) and autoradiographic techniques (Young and Kuhar 1979).

Ligand - receptor interactions are governed by the equilibrium:

$$L + R \leftrightarrows L\text{-}R \rightarrow \text{response}$$

where L = ligand, R = receptor, L-R = ligand-receptor complex (Clark and Peck 1980). If ligand and receptor are at equilibrium, then

$$B = nF/(K_d + F) \qquad \text{(equation 1)}$$

where B = mol of ligand bound, F = mol of free or unbound ligand, n = number of binding sites, K_d = dissociation constant = $1/K_a$, K_a = association constant.

Estimation of these parameters of ligand-receptor interactions has to be made at equilibrium conditions. Therefore, assays for receptor-hormone complexes are of limited use if the half-life of such a complex is shorter than the time required for the assay (Stoddart and Venis 1980).

Under equilibrium conditions free hormone may bind to two types of binding sites: a limited number of receptors with high affinity and specificity, and a second class, the nonspecific or background binding sites which have low affinity but large binding capacity (Clark and Peck 1980). To allow characterization of the specific and nonspecific binding components, total (= specific + nonspecific) binding is determined at a range of concentrations of radioactive hormone. Nonspecific binding is usually measured in parallel incubations using the labeled ligand in the presence of a large excess (~100X) of unlabeled competitor. As specific binding is

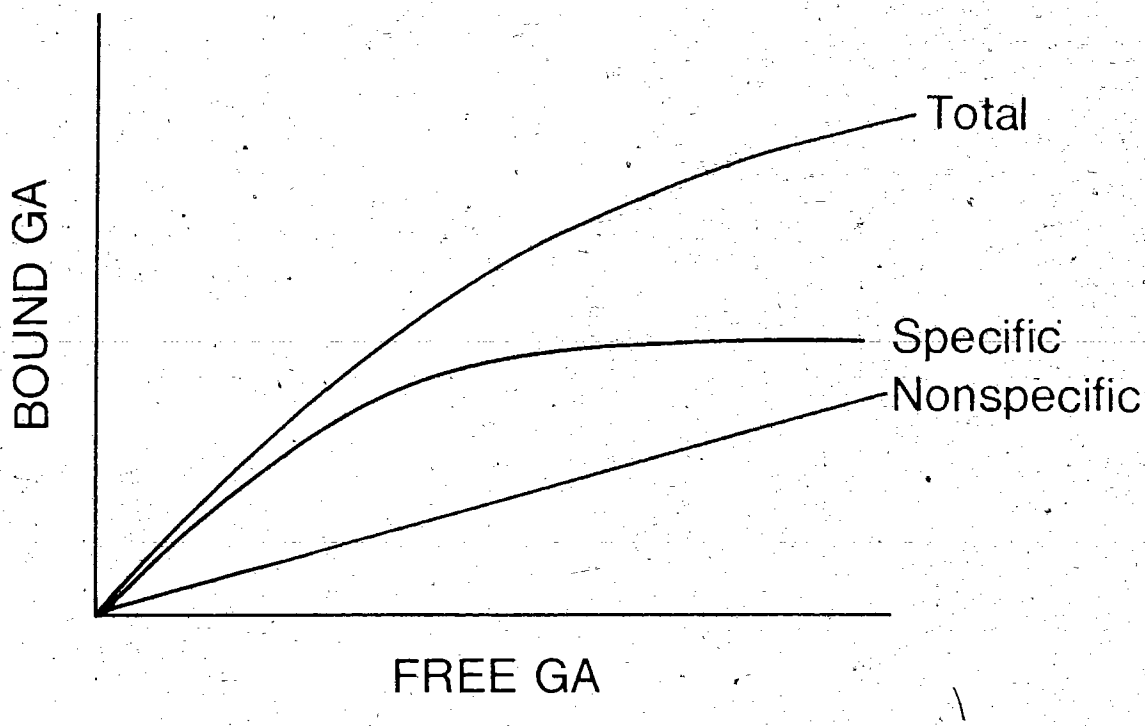
reversible, the unlabeled ligand occupies essentially all of the high affinity and saturable binding sites but does not alter the binding of the radioactive hormone to nonspecific sites. If specific binding is relatively large compared to nonspecific binding, simple one point assays for receptor binding can be performed by measuring total and nonspecific binding at a labeled hormone concentration equivalent to the estimated K_d of the receptor in the presence and absence of an excess of unlabeled competitor, respectively. A typical graph expected for a binding system containing one receptor is shown in Figure B2.a.

A number of mathematical transformations of equation 1 have been developed to allow a graphical estimation of those binding parameters that govern receptor-ligand interactions (e.g., Chamness and McGuire 1975, Rosenthal 1967, Scatchard 1949). The most commonly used is the 'Scatchard plot' using

$$B/F = -1/K_d\ (B - n) \qquad \text{(equation 2)}.$$

By plotting the measured values of B/F versus B, n, K_d or K_a can be estimated, as shown in Figure B2.b.

Figure B2. Analysis of interactions between GA molecules and GA binding components in an idealized system.
a) Binding to specific and nonspecific sites. The quantity of specifically bound GA is determined by subtracting nonspecific from total binding. b) Determination of receptor binding parameters using a Scatchard plot.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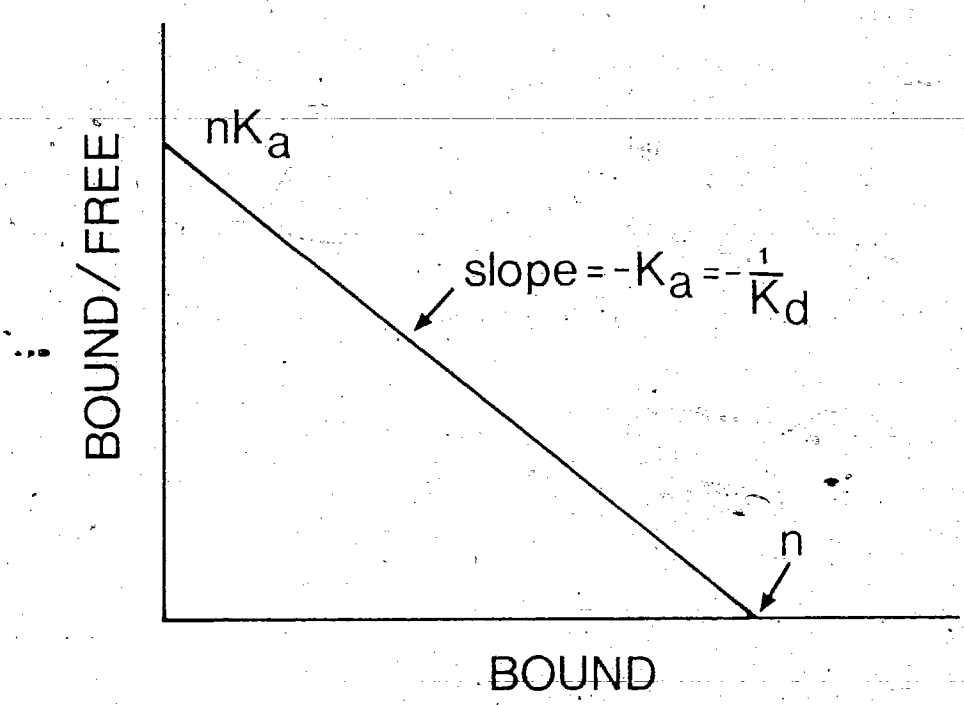
Total
Specific
Nonspecific
FREE GA


BOUND/FREE
nKa
slope = -Ka = -1/Kd
n
BOUND

IV. Evidence for the Existence of GA Receptors

Several main approaches have been used in GA receptor studies. In the first, GA-responsive plants are treated with radioactive GA *in vivo*. After varying periods of exposure the inter- and intracellular distribution of radioactivity is measured by autoradiography or by analysis of tissue extracts following homogenization and fractionation. Receptor-hormone complexes with a short half-life may not be detected by this method. Differences in uptake and transport rates, as well as metabolism of the applied GAs complicate such analysis of receptor - hormone interactions. The second approach used in locating GA receptors involves the *in vitro* measurement of the association of a labeled GA with subcellular fractions after cell fractionation. This approach assumes that monitoring GA action is not dependent on cellular integrity. Support for such a notion comes from animal receptor studies. With the third approach, identification of those cellular components that are required for GA-induced changes in RNA or protein synthesis is attempted.

Early support for the existence of GA receptors came from the work of Johri and Varner (1968). They demonstrated that isolated pea shoot nuclei show quantitative and qualitative changes in RNA synthesis only if GA is present during extraction and purification of the nuclei. Enhancement of RNA synthesis was greatest when 10 nM GA_3 was added at the beginning of the

purification and declined when GA_3 was added at progressively later steps in nuclear purification. The physiologically inactive GA_8 had no effect on RNA synthesis. The GA_3-enhanced RNA fraction was not purified and its relevance for *in vivo* growth was not investigated. Purification of the soluble factor required in GA stimulation of RNA synthesis does not appear to have been accomplished.

With the objective of finding particulate GA binding sites in pea stems Ginzburg and Kende (1968) investigated the intracellular localization of $[^3H]GA_1$ following an *in vivo* 24 h incubation. Microautoradiography showed that label is randomly distributed throughout the cells. More than 99% of the recovered radioactivity remained in the supernatant after differential centrifugation of the homogenates. The major part of the pelleted hormone was associated with intracellular membrane fractions. This binding to membranes was noncovalent but was not saturable. Metabolism of the ligand was not excluded, nor was the possibility that exchangeably bound GA_1 may dissociate from receptor sites. Abscisic acid (ABA) did not compete for binding.

In studies of *in vivo* GA binding Musgrave and coworkers were unable to provide clear evidence for the presence of GA receptors. Measuring the uptake of radioactive GAs by dwarf pea shoots, Musgrave *et al.*, (1969) observed that uptake of $[^3H]GA_1$ and $[^3H]GA_5$ by stem sections increases under conditions which would result in increased growth promoting activity. Thus accumulation of labeled GAs was higher in dark-grown, GA

responsive apical stem sections than in light-grown, GA insensitive basal shoot sections. Biologically inactive GA derivatives did not accumulate as much as active ones. The uptake of labeled GA was, however, not saturable. In a further study of GA uptake in barley aleurone layers Musgrave et al., (1972) noted that the accumulation of [^{3}H]GA_1, [^{3}H]GA_5, and [^{3}H]GA_5 methyl ester is not correlated with their biological activity but with their degree of metabolism. While binding was not saturable at 25 C, the authors did not examine saturability under conditions of inhibited metabolism, such as low temperature or in the presence of metabolic poisons which in their study reduced GA uptake. In the absence of metabolism, equilibrium between receptor and hormone could have become established along with receptor saturation.

Asakawa et al., (1974) applied [^{3}H]GA_3 of unspecified purity and specific activity to bean seedlings over a 24 h period to analyze translocation and intracellular distribution of the hormone. Plant tissues were then homogenized and fractionated to identify the location of GA. Most of the applied radioactivity (78.5 to 108.8(!)%) was associated with the 100,000g supernatant, very little (6 to 9%) with crude nuclear pellets. While failing to consider metabolism of [^{3}H]GA_3 or the possibility of dissociation of the growth regulator from its binding site, the authors were unable to detect association of radioactivity with proteins, DNA, or RNA.

Using nonaqueous extraction procedures for the cell fractionation of wheat endosperm, Jelsema et al., (1977) detected GA_1 binding sites in a particulate fraction enriched in aleurone grains. Binding of GA_1 was specific and could not be competed for by GA_8 but was by ABA. Specific binding was suggested to be reversible, with a K_d = 1.5 μM and 0.45 pmol binding sites per mg protein. However, in plotting their Scatchard diagram the authors used a logarithmic scale for their abscissa and thereby overestimated the values of n and K_d by 2 orders of magnitude. No apparent reason was given for choosing endosperm as the source for the extraction. The lack of extractable specific binding when aqueous procedures were used for the purification of the aleurone grains was also not explained.

Stoddart et al., (1974) incubated excised dwarf pea epicotyls with [^{3}H]GA_1 of unspecified purity for 12 h at room temperature. The 20,000g supernatant of homogenates of these sections was then examined for GA binding by passing it through a molecular sieve column and measuring radioactivity in eluate fractions. [^{3}H]GA_1 binding by high and intermediate molecular weight proteins was reversible and pH dependent, the latter fraction showing higher affinity binding. Equilibrium dialysis assays confirmed these results and indicated that GA-binding is specific. [^{3}H]GA_1 was not competed for by the inactive GA_8 and 3-epi-GA_1 but was by the growth promoting GA_1 and [^{3}H]-16-keto GA_1. No saturation of binding was shown. Metabolism of applied

hormones during the 12 h incubations was observed.

Konjevic et al., (1976) also investigated dwarf pea for GA binding proteins. The 10,000g supernatant from epicotyl homogenates was incubated with [^{14}C]GA_3 of unspecified purity. This extract was passed through molecular sieve and anion exchange columns and the radioactivity in column eluates measured. An $(NH_4)_2SO_4$ precipitation assay was also used. The results obtained failed to indicate receptor-type binding. Saturation of binding sites was not shown and is unexpected as the authors were working with very crude extracts and performed binding studies at room temperature. Dissociation of GA from the binding sites was not taken into account. Furthermore, binding was not specific as GA_4/GA_7 and $GA_{1,3}$ were equally effective in displacing about 30% of the bound radioactivity. Protease inhibitors were not used although extraction procedures were at room temperature. Thus the risk of receptor degradation was not reduced.

In vivo uptake of [^{3}H]GA_1 by lettuce hypocotyls was observed by Stoddart (1979) to correlate with rates of GA promoted cell elongation. Hypocotyls were incubated at 28 C for 24 to 48 h and bound radioactivity measured. Of the total absorbed radioactivity, 95% was associated with the cytosol while 4% was bound by a cell wall containing fraction pelleted at 2,000g. This pelleted binding was hypothesized to correlate with GA induced changes in cell wall plasticity. The binding was observed to be unsaturable, covalent and temperature dependent.

As experiments were performed at or above 10 C, metabolism of the supplied [^{3}H]GA$_1$ was observed.

On the basis of the GA binding studies discussed to this point Kende and Gardner (1976), Venis (1977), Rubery (1981), and Stoddart and Venis (1980) concluded that there is little evidence in support of the presence of GA receptors. However, Keith et al., (1980) reinvestigated this problem with barley aleurone layers. They measured saturable binding of [^{3}H]GA$_1$ to the layers when metabolism of the hormone was stopped with incubations at 1 - 1.5 C. At temperatures above 3 C metabolism occurred and no saturation of binding could be detected. Saturating concentrations of GA$_1$ but not GA$_8$ could displace bound radioactivity.

Saturable in vivo binding of GAs to cytosol of dwarf pea epicotyls was demonstrated by Keith and Srivastava (1980) who, in contrast to the earlier work by Stoddart et al., (1974) used incubations at 0 C to stop metabolism and sliced sections to reduce transport and permeability effects. Under these conditions they were able to detect 2 classes of binding sites in gel filtration column eluates, one with high affinity (Kd = 60 nM, n = 0.4 pmol·g^{-1} fresh weight). [^{3}H]GA$_1$ binding could be inhibited by biologically active but not by inactive GAs or other plant hormones. Binding was susceptible to protease treatment but was irreversible when assayed in vitro using gel filtration columns. Stoddart (1982) considered the number of these high affinity binding sites to be too low to be of

physiological significance. However, he failed to account for loss of binding sites due to the extraction procedure and underestimation of binding sites with the nonequilibrium assay.

With an approach similar to that used in pea, Keith et al., (1981) were able to demonstrate in vitro binding of [^{3}H]GA_4 to cucumber hypocotyl cytosol using the gel filtration column assay. GA_4 binding to soluble proteins was shown to be reversible, saturable and of high affinity. DNAse, RNAse, protease and heat treatments demonstrated that specific binding of GA_4 was to proteins. A good correlation between in vitro binding affinity and in vivo growth promotion was observed for a series of GAs. With the development of a very rapid DEAE filter paper assay (Keith et al., 1982), the results obtained with cucumber could be confirmed and refined. The authors observed a single class of binding sites with a K_d = 70 nM for GA_4, n = 0.7 pmol·g^{-1} fresh weight, half-time of dissociation = 6 min for GA_4. Other biologically active GAs competed for the same binding sites as [^{3}H]GA_4 while biologically inactive GAs did not bind. Specific binding was also detected in GA-nonresponsive basal sections of hypocotyls or cotyledons. The concentration of binding sites was the same as in the GA-responsive apical hypocotyl portions on a mg protein basis. However, on a fresh weight basis the apical target tissue had greater amounts of specific binding. Assays using resuspended 130,000g pellets and extracts using Triton X-100 supported the view that specific binding sites are on soluble proteins.

Further support for these *in vitro* results has been recently provided by studies using etiolated dwarf pea: Lashbrook *et al.* (1987) used the Sephadex G-100 assay to demonstrate saturable, exchangeable *in vitro* binding of [^{3}H]GA_1 to an intermediate molecular weight fraction from cytosol. Two pH optima for binding were observed. However, *in vitro* specificity data were inconclusive. Liu and Srivastava (1987) used the DEAE filter paper assay and [^{3}H]GA_4 for the measurement of GA binding proteins (GABPs) in pea cytosol fractions. They observed saturable, exchangeable binding of [^{3}H]GA_4 with a K_d near 100 nM. There was a correlation between *in vivo* activity and *in vitro* affinity of the protein for GA_4, GA_3, and GA_4 methyl ester. Scatchard plots suggested similar numbers of binding sites in dark-grown tall (cv. Alaska) and dwarf (cv. Progress No. 9) peas. This should be expected, as these cultivars show similar growth habits and GA sensitivity in the dark. The dwarf phenotype and high GA sensitivity of Progress No. 9 are expressed in light, presumably due to decreased enzymatic conversion of GA_{20} to GA_1 and possibly also compartmentation of GA_1 (Campell and Bonner 1986, Sponsel 1986).

Similar efforts by Keith and Rappaport (1987) to show receptor-type binding in maize mutants were, however, unsuccessful. Although [^{3}H]GA_1 binding to cytosol was pH sensitive, it was largely unspecific and nonexchangeable at the 2 pH optima. The effect of pH on GA stability (see review by Takahashi *et al.* (1986) was not taken into consideration. Lack

of receptor stability appeared to have contributed to these results.

V. Conclusions

The results from the laboratories of Rappaport and Srivastava provide strong evidence for the existence of GA receptors in a number of different plant systems. With the pea epicotyl and the cucumber hypocotyl systems many of the criteria expected of GA receptors are fulfilled. Thus GA binding is saturable, reversible and specific with a high affinity for biologically active GAs, whereas GAs that do not promote growth do not bind. The development of the in vitro DEAE filter paper assay has led the way to allow characterization of GA structure/activity relationships in the absence of metabolism and permeability barriers. One can, therefore, determine which GA(s) among the many found in a plant, is actually controlling development.

The GA binding assay also permits examination of tissue extracts for GA binding activity and thereby is of great importance in purification of putative GA receptors. With a purified receptor the site(s) of its action and its role in plant development can be studied.

PART C

In Vitro Assay of Gibberellin Binding Proteins (GABPs)

Keith et al. (1982) developed the DEAE filter paper assay for the quantitation and characterization of GA--binding protein complex formation. Their work was repeated and some modifications introduced. These changes and some results obtained with other assay methods are described in this chapter.

I. Materials and methods

1. Plant material

Following the procedure of Keith et al. (1982) seeds of cucumber (Cucumis sativus L. cv. National Pickling, Buckerfields & Co., Vancouver, B. C.) were surface sterilized in 50% household bleach, 5 min, and sown in moist, autoclaved vermiculite in flats. Seedlings were grown in darkness at 28 C, 6.5 days, then exposed to fluorescent light (10 $\mu E \cdot m^{-2} \cdot sec^{-1}$) in the laboratory for 14 h.

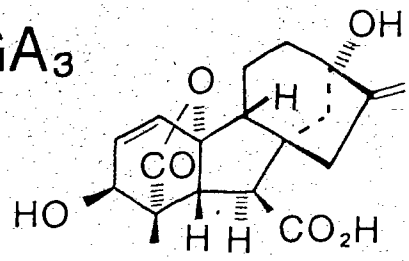
2. Gibberellins and other compounds

Structures of the GAs discussed in this chapter are shown in Figure C1. The purity of some of the GAs was estimated by gas liquid chromatography (GLC) and high pressure liquid chromatography (HPLC) as described below.

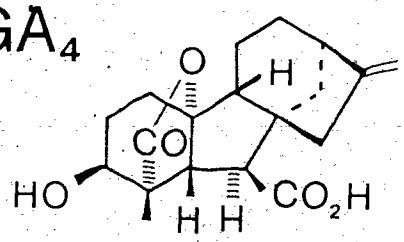
GA_4 and GA_7 were purchased from Abbott Laboratories, Chicago, IL. Their purity was estimated to be >95% and >90%, respectively. GA_3 (>89% purity) was purchased from Sigma. C-7 methyl ester of GA_4 (GA_4ME) was synthesized by Zin Liu or me in our lab by methylation of GA_4 using ethereal diazomethane and purified by thin layer chromatography (TLC) to greater than 95% purity (estimated by HPLC). No GA_4 was detected in this preparation. [^{3}H]GA_4, prepared by catalytic reduction of GA_7 and

Figure C1. Structures of GA_3, GA_4, GA_7, and GA_4 methyl ester.

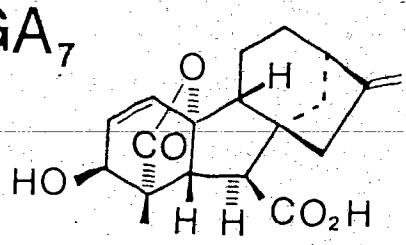
GA_3



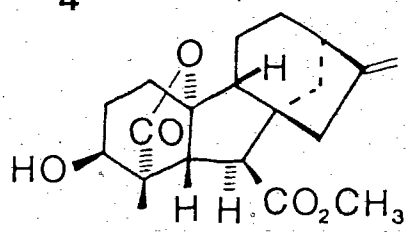
GA_4



GA_7



GA_4ME



presumably labeled at C-1 and C-2 (tritiation by the palladium-catalyzed actions of ^3H gas on GA_7 was performed by NEN, Boston, MA), was purified in our laboratory in cooperation with Zin Liu by preparative (Silica Gel G, 1000 μm, Analtech Inc., Newark, DE) and analytical TLC (Silica Gel 60, 0.25 mm E. Merck, Darmstadt, F.R.G.). The organic phase of benzene, acetic acid, water (8;3;5, v/v) mixed with acetic acid (14:1, v/v) was used as the solvent. The purity of the [^3H]GA_4 was estimated by GLC to be greater than 65%, about 5% contamination was due to precursor GA_7 and the rest due to unknown products (Fig. C2). The radiochemical purity was estimated by combined HPLC-radioactivity counting (HPLC-RC) to be 61% (Fig. C3.a). The mass of the [^3H]GA_4 was estimated by comparing the peak area in GLC spectra against those of known concentrations of GA_4. The specific activity of [^3H]GA_4 was estimated by liquid scintillation counting of aliquots of [^3H]GA_4 of known mass and calculated to be 1.6×10^{12} Bq mmol^{-1}. GLC-MS spectra of the products purified from model hydrogenation and deuteriation reactions of GA_7 suggested that the final product is indeed [^3H]GA_4 and that the C_{16}-C_{17} methylene group was not saturated (Z.H. Liu, pers. comm.).

For some experiments [^3H]GA_4 with 1.4×10^{12} Bq mmol^{-1} and 98.1% radiochemical purity (Fig. C3b) was used. This product, sold by Amersham Canada Ltd., Oakville, ON, resulted in lower nonspecific binding (see 'Results') when used in the DEAE filter paper assay. It became available during the last stages of this

Figure C2. GLC elution profile of [^{3}H]GA_4 partially purified from NEN reaction products. Peaks eluting at the retention times of GA_4 and GA_7 are marked. Substances eluting prior to '*' are contaminants arising from the derivatization compounds used and also appear in control samples containing no GAs.

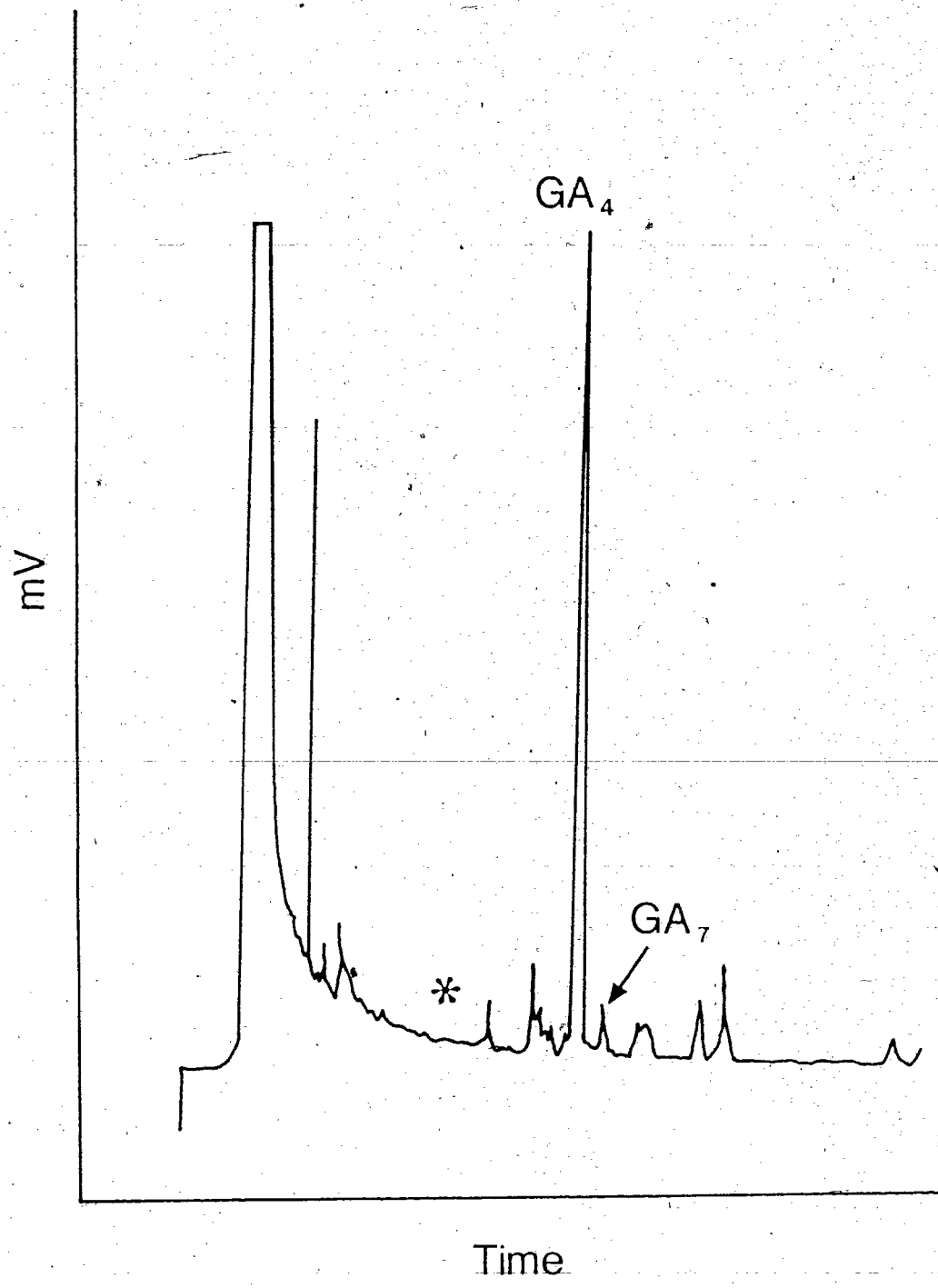
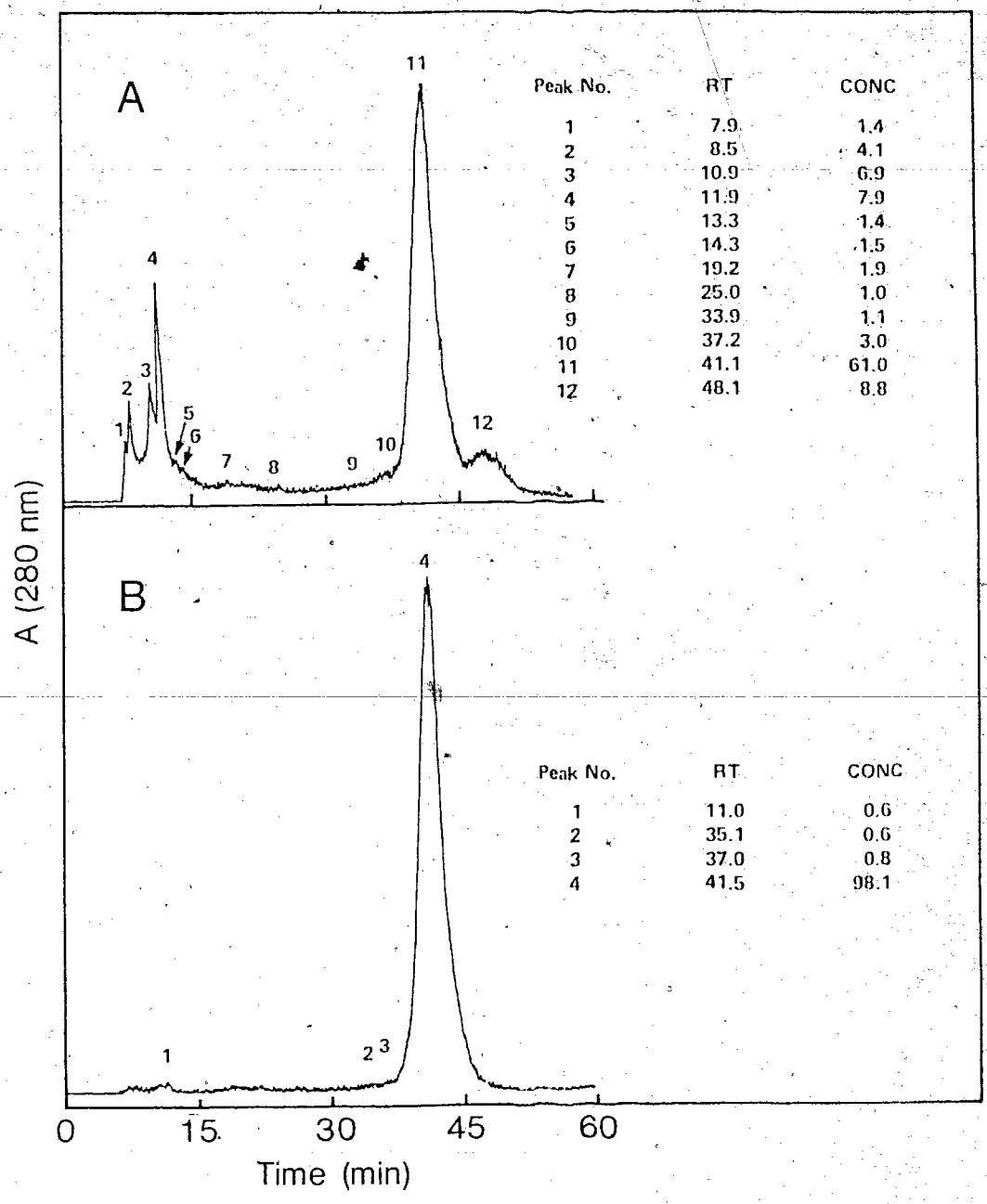

GA4
GA7
*
mV
Time

Figure C3. HPLC-RC elution profiles of [^{3}H]GA_4 partially purified from NEN reaction products (a) and as supplied by Amersham (b).


A
B
A (280 nm)
Time (min)
0
15
30
45
60
1
2
3
4
5
6
7
8
9
10
11
12
Peak No. RT CONC
1 7.9 1.4
2 8.5 4.1
3 10.9 6.9
4 11.9 7.9
5 13.3 1.4
6 14.3 1.5
7 19.2 1.9
8 25.0 1.0
9 33.9 1.1
10 37.2 3.0
11 41.1 61.0
12 48.1 8.8
Peak No. RT CONC
1 11.0 0.6
2 35.1 0.6
3 37.0 0.8
4 41.5 98.1

study only. Most critical experiments were redone with Amersham [^{3}H]GA_4 and confirmed the results obtained with the product from NEN. GAs were stored in ethyl acetate, ethanol (1:1, v/v) at -20 C. All other chemicals used were of analytical grade.

3. GLC of GAs

Methylated and trimethylsilylated derivatives of the GAs were prepared using ethereal diazomethane for methylation and TriSil Z (Pierce Chemical Co., Rockford, IL) for trimethylsilylation according to the method of Binks et al. (1969). A Hewlett Packard 5790A gas liquid chromatograph was used with a fused silica capillary column (SE-30, 0.2 mm x 12.5 m, J.&W. Scientific) injection temperature: 260 C, column temperature: 250 C, detector temperature: 280 C.

4. HPLC of GAs

Purity of GAs was also monitored by HPLC. A preparative C-18 reverse phase column (Magnum 9, Partisil 10, ODS 2, Whatman) was used with a Waters gradient chromatography system coupled to a recorder-integrator. Buffer A, 100% MeOH; buffer B, 1% acetone in water adjusted to pH 2.7 with HCl. For chromatography of GA_3 40% buffer A, 60% buffer B was used; for GA_4, GA_7 60% buffer A, 40% buffer B. Absorbance at 206 nm was monitored. Under these conditions and a flow rate of 3 ml min^{-1}, GA_3 elutes after 22.5 min, GA_4 after 41.1 min, and GA_7 after 34.1 min. GA_4 and GA_4ME

were compared using 85% buffer A, 15% buffer B. Here GA_4 and GA_4ME elute after 9.7 min and 12.4 min, respectively (see also Jensen et al. 1986).

Purity of radiolabeled GAs was measured by HPLC-RC. A Ramona LS flow-through radioactivity monitor (Raytest Strahlungsmessgeräte GMBH, Straubenhardt, F.R.G.) was coupled to the HPLC. For analytical runs a split ratio of 9:1 with a scintillant (Atomlight, NEN) : column eluate ratio of 3:1 was used. Photons emitted from mixed sample and scintillant in a 2.1 ml measurement cell were measured by photon detectors. Static tritium counting efficiency was 38% under these conditions.

5. GAPB extraction

All procedures were performed at 0 to 3 C. Apical 1.5 to 2 cm of hypocotyls were cut and pooled into freshly prepared extraction buffer (100 mM Tris, 1 mM EDTA, 50 μM phenylmethylsulfonyl fluoride (PMSF), adjusted to pH 7.3 with H_3PO_4), drained and homogenized in an equal volume (1:1, w/v) of extraction buffer using mortar and pestle. The homogenate was passed through 2 layers of cheese cloth and centrifuged at 100,000g, 1.5 h. The supernatant cytosol was used for some GABP assays. For most experiments, $(NH_4)_2SO_4$ was added to this supernatant to 60%. After equilibration and centrifugation at 24,000g, 20 min, the pellet obtained was washed in column buffer, particulates removed at 7,000g, 5 min, and the

supernatant desalted on a column of Sephadex G-25 Fine (Pharmacia). The protein fraction eluted was then used for GABP assays and is called G-25 eluate. Protein measurements were made according to the method of Bradford (1976) using BioRad protein assay solution (BioRad, Richmond, CA) with BSA as a standard.

6. Incubations

High affinity specific binding of [^{3}H]GA_4 by the putative receptor has to be distinguished from low affinity, nonspecific binding by other macromolecules. For this purpose, buffer controls or protein fractions were typically incubated with 50 nM [^{3}H]GA_4 in the absence or presence of an excess of selected unlabeled GAs for 1.5 h. This incubation time was used by Keith et al. (1981) and was also confirmed by my preliminary experiments to be adequate for equilibration (data not shown). Metabolism of ligands was inhibited by keeping all solutions at 0 to 2 C (see Keith et al. 1980).

7. DEAE-Cellulose filter assay

The methodology used is based on that described by Keith et al. (1982). At neutral pH the GABP is negatively charged and binds to DEAE-cellulose filters. Ligands bound by the protein are retained while unbound molecules are washed through. Protein binding to filters is affected by cations, hence low ionic strength buffers are used for incubations as well as wash.

Stacks of 2 filter discs (2.4 cm diameter, Whatman DE81) moistened in ice-cold assay buffer (in early experiments: 10 mM Tris-HCl, 1 mM EDTA [pH 7.5], later: 10 mM K-phosphate, 1 mM EDTA [pH 7.0]) were placed on a vacuum filtration manifold (Hoefer 225 V). The filters were equilibrated with 25 ml assay buffer. The vacuum was released and aliquots of the incubation mixture pipetted onto the filters. After exactly 1 min the filters were washed with assay buffer to remove unbound ligand molecules. Sample and wash volumes are specified in 'Results'. The filter stacks were allowed to dry for about 5 sec, placed in scintillation vials and 1 ml absolute EtOH added. After 30 min 6 ml of scintillant (Scintiverse 2, Fisher) was added, the vials shaken and left in the dark before counting with a Beckman LS 8000 liquid scintillation counter at about 44% efficiency. The [^{3}H]GA_4 concentration in the incubation mixture was routinely determined by measuring the radioactivity of 10 μl unfiltered aliquots. Triplicate samples were used. Sample deviation from the mean was generally less than 10%.

8. In vivo assay

Cucumber seedlings were grown in flats for 6.5 days in the dark as already described. They were then transferred to a growth chamber at 2.5 $\mu E \cdot m^{-2} \cdot sec^{-1}$ 25 C for 14 h. At this stage the seedlings were about 5 cm tall and were selected for uniformity. The apical 1 cm of hypocotyls was marked with India ink. GAs were applied to each hypocotyl in 4 μl of absolute ethanol.

Controls received the solvent only. After 4 days in the growth chamber hypocotyl elongation was measured. Fifteen seedlings were used per treatment.

II. Results

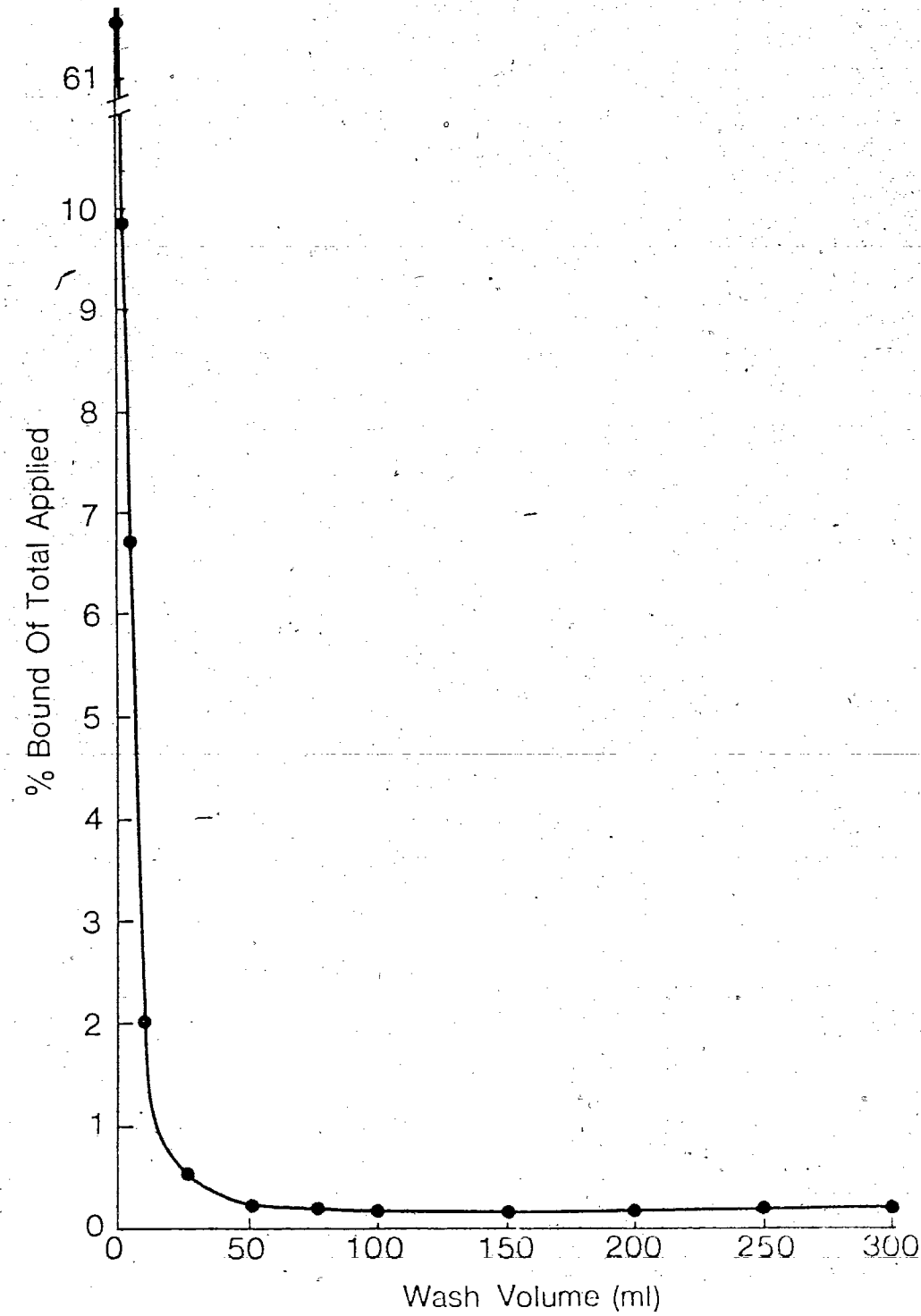
1. DEAE-Cellulose filter assay

Only low levels of specific binding were detected in cytosol fractions when filter paper assays were performed according to the procedure of Keith et al. (1982) (data not shown). As the batch of filter paper and [^{3}H]GA$_4$ available to me differed from that used by those authors it was decided to reexamine some of the basic parameters of the filter paper assay. Conditions for increasing the sensitivity of GABP detection were sought.

a. Wash volume

The purpose of this experiment was to determine the volume of assay buffer wash required to separate free from bound [^{3}H]GA$_4$ on the filter paper. The results shown in Figure C4 indicate that at least 50 ml assay buffer are required to efficiently remove free [^{3}H]GA$_4$ from the filter discs. Of the total radioactivity applied to the filter stack, 0.2% is adsorbed and cannot be washed off (see also Keith et al. 1982). A wash volume of 100 ml was chosen to permit small errors in volume delivery without affecting the extent of removal of unbound ligand. Exceeding the 100 ml wash or inclusion of 0.1 M KCl in the assay buffer results in a decrease of detectable [^{3}H]GA$_4$ binding to the GABP (data not shown). This could be due to increased dissociation of [^{3}H]GA$_4$ from the binding sites and/or desorption of protein from the filter paper during the

Figure C4. Effect of wash volume on [^{3}H]GA_4 binding to DEAE filter paper.
50 μl aliquots of incubation mixture containing 50 nM [^{3}H]GA_4 were loaded onto filter paper stacks. After 1 min suction was applied and the filters washed with different volumes of assay buffer. Data shown are the average of 2 experiments. Total radioactivity applied = 79,000 cpm · 50 μl^{-1}.


61
10
9
8
7
6
5
4
3
2
1
0
% Bound Of Total Applied
0
50
100
150
200
250
300
Wash Volume (ml)

lengthier wash. The 100 ml wash requires about 1 min, while the half-life of dissociation of GA_4 from the binding site is less than 10 min (Keith et al. 1982), and Part D this report). Therefore, hormone binding to protein is not measured at equilibrium and hence specific binding will be underestimated.

b. Sample and assay buffer pH

The binding of [^{3}H]GA_4 to the filter paper in the absence of cytosol, as well as total binding in its presence, were measured at incubation and assay buffer pH values ranging from 6.75 to 8.0. Specific binding was determined by subtracting nonspecific binding, measured in the presence of a 100 fold excess of unlabeled GA_4, from total binding. Figure C5 shows that the radioactivity adsorbed to the filter paper in the presence or absence of cytosol decreases as pH increases. Small changes in pH at pH 7.5 have a significant effect on total and specific binding. Moreover, the difference between specific and background binding is increased below pH 7.0. For these reasons sample and assay buffers were maintained at pH 7.0 for all further experiments.

c. Sample volume and protein concentration

Figure C6 shows that total and specific binding of [^{3}H]GA_4 is linear for sample volumes ranging from 25 to 200 μl when a cytosol preparation containing 2 mg protein$\cdot$ml^{-1} is used. [^{3}H]GA_4 binding to a desalted, resuspended 60% $(NH_4)_2SO_4$ precipitate of the cytosol was assayed in the absence and

Figure C5. Effect of pH on [^{3}H]GA_4 binding to DEAE cellulose filter paper.
Aliquots of incubation mixture containing extraction buffer in the presence or absence of cytosol were assayed at the pH shown. Specific binding was determined by subtracting nonspecific binding from total binding. Cytosol was assayed at 1 $mg \cdot ml^{-1}$.

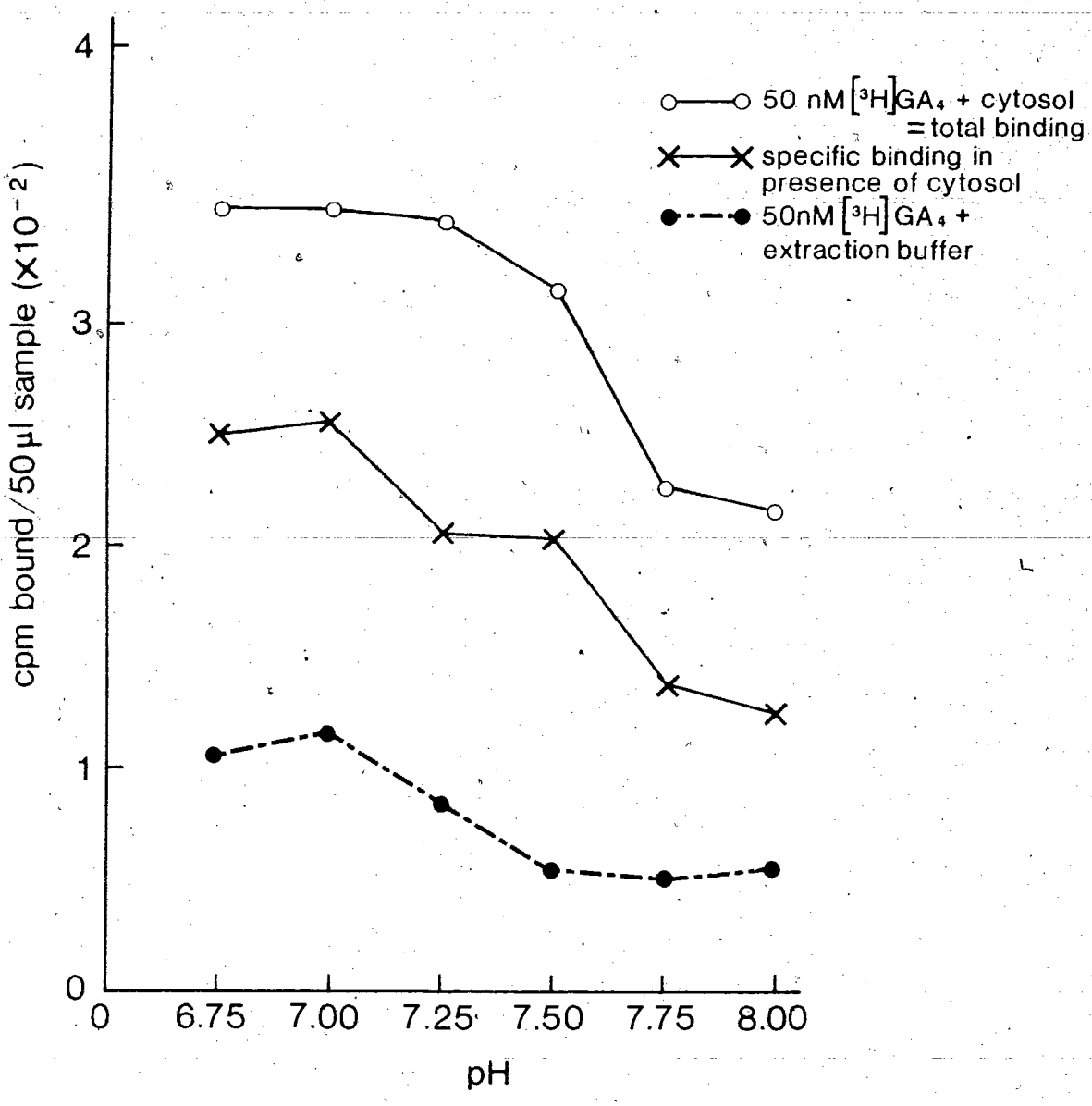
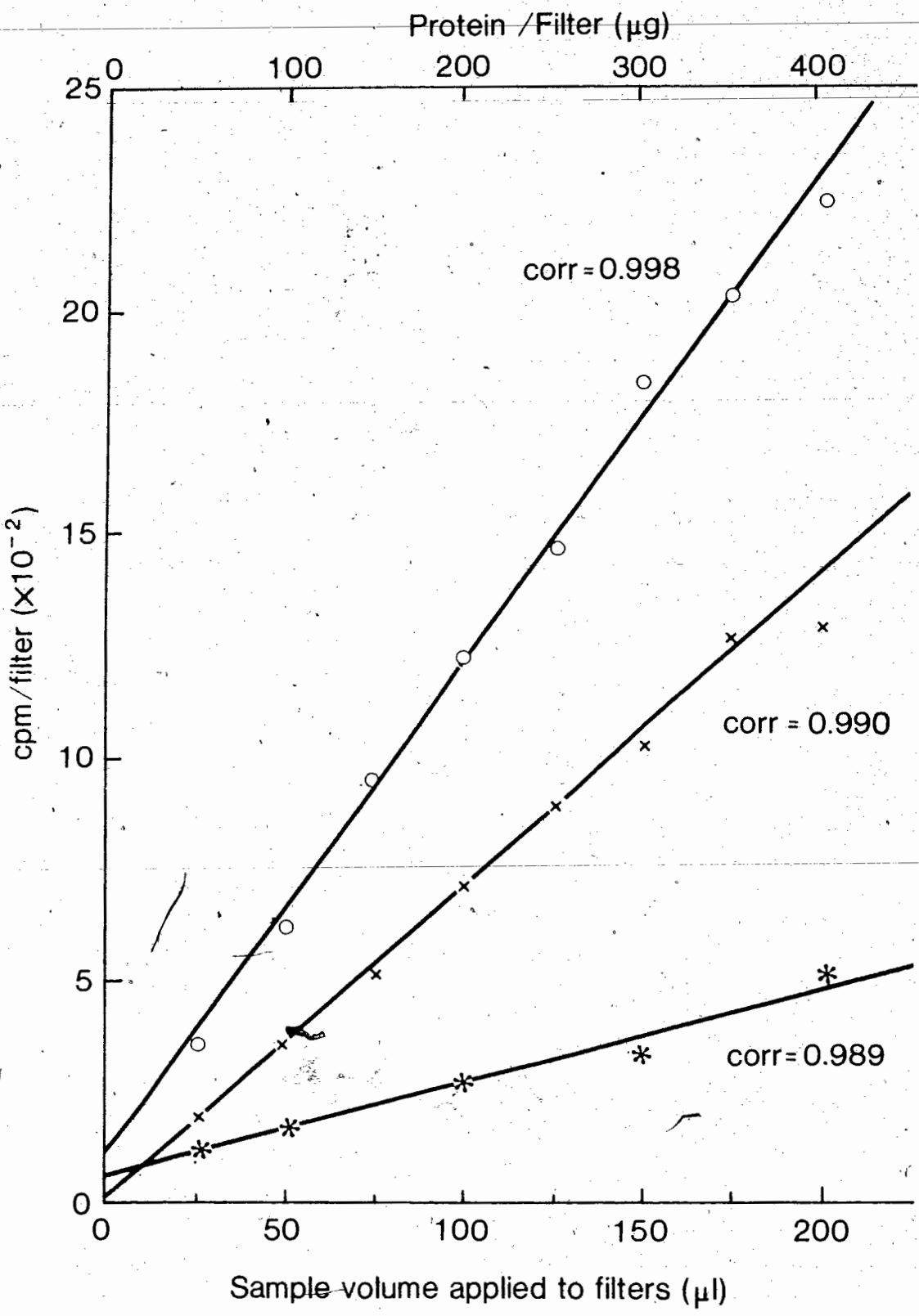

4
3
2
1
0
0
6.75
7.00
7.25
7.50
7.75
8.00
pH
cpm bound/50µl sample (X10⁻²)
50 nM [³H]GA₄ + cytosol
= total binding
specific binding in
presence of cytosol
50nM [³H] GA₄ +
extraction buffer

Figure C6. Effect of sample volume on [^{3}H]GA_4 binding to filter paper. Cytosol at 2 mg protein·ml^{-1} was used for the determination of total (o) and specific (x) binding. Binding in the absence of cytosol (*) was also measured.


Protein /Filter (µg)
0
100
200
300
400
25
20
15
10
5
0
cpm/filter (X10⁻²)
corr = 0.998
corr = 0.990
corr = 0.989
0
50
100
150
200
Sample volume applied to filters (µl)

presence of an excess of GA_4. As shown in Table C1, the sample volume used does not greatly influence results when data are presented on a $cpm \cdot ml^{-1}$ or $cpm \cdot mg^{-1}$ protein basis. This at least is valid for samples containing high protein concentrations as found in the G-25 eluate. Table C1 also indicates that in the absence of protein unlabeled GA_4 can compete for a substantial number of the $[^3H]GA_4$ binding sites on the filter paper. This 'apparent specific' binding, therefore, contributes to the specific binding observed with the cucumber extracts. By loading >100 µg protein per sample the proportion of the specific binding to the receptor is kept in sufficient excess over apparent specific binding to the filter paper.

d. GA binding by nonreceptor components

The results in Table C1 indicated that unlabeled GA_4 competes for $[^3H]GA_4$ binding sites on the ion exchange filter paper. This, of course, makes detection of GABP containing fractions difficult. In order to resolve the difference between 'apparent specific' and 'specific' binding a selection of unlabeled GAs with different *in vivo* activity were tested for competition with $[^3H]GA_4$. These were used in incubation mixtures containing column buffer, column buffer + BSA, and G-25 eluate. GA_4 with high, GA_3 with moderate, and GA_4ME with little or no *in vivo* activity were used as competitors. The results (Table C2) indicate that ability for a competing GA to displace radioactivity from the filter paper correlates with *in vivo* growth promotion only in the cucumber extract. Data on *in vivo*

Table C1. Effect of sample volume on GA_4 binding to filter discs in absence and presence of G-25 eluate. Resuspended, desalted 60% $(NH_4)_2SO_4$ precipitate of cytosol (3.5 mg protein·ml^{-1}) of column buffer was incubated for 1.5 h with 50 nM [^{3}H]GA_4 in the absence and presence of 5 μM GA_4. Aliquots of the incubation mixture were assayed as described in 'Materials and Methods'. Total, nonspecific, and 'specific' binding were determined as decribed in Figure C5. Data represent cpm bound per sample.

Sample	Sample Vol. (μl)	Total	Nonspecific	'Specific'
Column buffer	50	390	190	200
	100	1,030	440	590
G-25 eluate	50	1,000	500	500
	100	1,910	970	940

Table C2. Competition for [^{3}H]GA_8 binding in incubation mixtures containing column buffer, G-25 eluate or BSA. Data represent cpm bound per 100 µl sample.

Incubation	*in vivo* activity	column buffer	G-25[a] eluate	BSA
50 nM [^{3}H]GA_8		380	1,450	14,580
+ 5 µM GA_8	+++	230	790	7,710
+ 5 µM GA_3	++	200	1,060	14,640
+ 50 µM GA_8ME	0	210	1,260	13,860

[a]G-25 eluate was assayed at 4.2 and BSA at 2.8 mg protein·ml^{-1}

biological activities of these GAs and GA derivatives are shown in Table C3. The data from these two tables reinforce the notion, therefore, that detection of GABP containing fractions requires the use of a number of GAs in binding assays in order to discriminate against nonreceptor GA-binding sites.

The possibility that some of the radioactivity adsorbed by the filter paper and protein is not [^{3}H]GA_4 but results from unspecified contaminants present in the [^{3}H]GA_4 purified in our laboratory to 61% radiochemical purity was also investigated. For this purpose [^{3}H]GA_4 supplied by Amersham with 98.1% radiochemical purity (Fig. C2) and a specific activity of 1.4×10^{12} Bq·mmol^{-1} was used. The results shown in Table C4 show that less radioactivity is bound from samples containing Amersham's [^{3}H]GA_4 and that the inactive GA_4ME displaces less radiolabel from binding sites in G-25 eluate compared to samples containing the [^{3}H]GA_4 purified in our laboratory. The lower background radioactivity does improve binding data. The Amersham product became available only in the last stages of this study. With it the general trends observed with the product from NEN could be confirmed. binding studies.

The results in Tables C1 and C2 demonstrated that [^{3}H]GA_4 is bound by DEAE filter paper and that this binding is competed for by unlabeled GAs. It was attempted to reduce this adsorption of GAs by soaking the DEAE filters in assay buffer containing 10 μM GA_4 and thereby saturate all binding sites. Table C5 shows that this treatment had an insignificant effect and did not improve

Table C3. In vivo cucumber hypocotyl assay using GA_4, GA_3 and GA_4ME.
Test compounds were applied in 4 µl 100% ethanol. The length of the apical 1 cm of hypocotyls was measured after 4 days. Dosages given as mass/plant. Values with different superscripts are significantly different at the 95% confidence level (Duncan's multiple range test) (Puri and Mullen 1980).

Treatment	Hypoctyl Length (cm)
Control	1.1^{a}
1 µg GA_4	1.9^{b}
1 µg GA_3	1.3^{c}
1 µg GA_4ME	1.1^{a}
10 µg GA_4ME	$1.2^{a,c}$
100 µg GA_4ME	$1.2^{a,c}$

Table C4. Effect of [^{3}H]GA_4 from 2 sources on filter paper binding in presence and absence of G-25 eluate. Data represent cpm bound per 100 µl sample. G-25 eluate was assayed at 2.5 mg protein·ml.

Incubation	Column buffer	G-25 eluate
50 nM [^{3}H]GA_4[a]	760	2,490
+ 5 µM GA_4	410	1,680
+ 5 µM GA_3	360	2,070
+ 50 µM GA_4ME	360	2,100
50 nM [^{3}H]GA_4[b]	600	1,380
+ 5 µM GA_4	410	310
+ 5 µM GA_3	400	1,240
+ 50 µM GA_4ME	420	1,370

[a] [^{3}H]GA_4 prepared by NEN and partially purified in our laboratory

[b] [^{3}H]GA_4 supplied by Amersham in 1986

Table C5. Effect of using DEAE filters presoaked in GA_4 on $[^3H]GA_4$ binding in the presence and absence of G-25 eluate. Samples were loaded on DEAE filters that had been soaked in assay buffer in the absence (control) or presence of 10 μM unlabeled GA_4. Data represent cpm bound per 100 μl sample.

Sample	column buffer		G-25 eluate	
	control	GA_4-soaked	control	GA_4-soaked
50 nM $[^3H]GA_4$	820	780	1,930	2,170
+ 5 μM GA_4	350	290	1,010	990
+ 5 μM GA_3	360	320	1,280	1,230
+ 50 μM GA_4ME	340	330	1,400	1,540

the quality of the data when NEN [^{3}H]GA_4 was used. Similar results were obtained with Amersham [^{3}H]GA_4. Although radioactivity bound to the filter paper was close to background. The basis for these results is not apparent.

III. Discussion

The interaction between GA, GABP and other macromolecules is complex. In order to distinguish binding sites of the receptor from others, a rapid and reliable assay system is required. Keith et al. (1982) had introduced the DEAE-cellulose filter paper assay for GABPs. That method was shown to be more efficient than the Sephadex G-50 column assay (Keith et al. 1981). Among the methods I tested (see APPENDIX 2), the DEAE filter assay was also shown to be the method of choice.

On the basis of my experiments some minor changes to the procedure of Keith et al. (1982) were introduced to enhance the difference between measured 'specific' and 'nonspecific' binding. Thus the volume of assay buffer, used to remove the free ligand from the filter paper, was increased from 75 ml to 100 ml and its pH changed from pH 7.5 to 7.0.

While these changes result in some improvement of data quality they also have some drawbacks. Thus by increasing the wash volume the likelihood of GABP being washed off the filter paper and the possibility of increased GA - GABP dissociation is enhanced. Furthermore, Keith et al. (1981) have shown that binding of [^{3}H]GA_4 to the GABP has an optimum at pH 7.5 when assayed using Sephadex G-50 columns. In that assay system separation of bound from free ligand is not likely greatly affected by sample pH. With the DEAE filter assay used here small changes in pH near pH 7.5 appear to have a significant

effect on the protein binding capacity of the filter paper. A pH of 7.0 was chosen for the DEAE filter assay because the difference between specific and background binding is enhanced and small changes in pH have little effect on [^{3}H]GA_4 bound (Figs. C4, C5). The choice of pH and wash volume will, therefore, result in an underestimation of the number of receptor binding sites. Further limitations to the assay are imposed by the purity of the radiolabeled GA, as evidenced by the comparison of the partially purified NEN [^{3}H]GA_4 with the Amersham product (Table C4).

Displacement of [^{3}H]GA_4 by GA_4 occurs not only from the binding site of GA receptor-like proteins, but also the DEAE filter paper and nonreceptor proteins such as BSA (Table C2). These nonreceptor entities contribute to 'apparent specific' binding. Receptor-type specific binding therefore can not be determined by simply subtracting nonspecific binding in the presence of an excess of unlabeled GA_4 from total binding. In order to screen tissue extracts for the presence of receptor-type proteins a more rigorous assay involving a series of biologically active and inactive competitors is required. Such assays may not be able to discriminate between [^{3}H]GA_4 binding to receptor-like proteins and binding to enzymes of GA biosynthesis or metabolism (eg., hydroxylases or glycosylases).

PART D

Characterization of GA Binding Sites

With the DEAE filter paper assay, data are obtained at 0 to 3 C when metabolism of GAs is almost nonexistent (Keith et al. 1980). The possibilities of conversion of the GA or GA derivative supplied to a different form during the assay are therefore negligible. Furthermore, since the assays are performed in vitro using cytosol, the concentration of the GAs used reflect the concentration at the binding sites. The filter assay thus allows a study of the affinity of the GA binding sites for different GAs.

Keith et al., (1981, 1982) have shown that the GABPs in cucumber cytosol bind GA_4 with high affinity (K_d = 70 nM). This binding is saturable (n = 0.4 pmol·mg^{-1} soluble protein) and exchangeable (half-life of dissociation = 6 to 7 min). Moreover, by double reciprocal plots they showed that GA_7 competes for the same binding sites as GA_4 but the inactive GA_{26} does not.

In this chapter I shall report on the binding of a series of biologically active and inactive GAs, GA derivatives, and other plant growth substances to a partially purified cytosol preparation. The results provide information on those features of the GA molecule that are important in GA--receptor protein interaction.

I. Materials and Methods

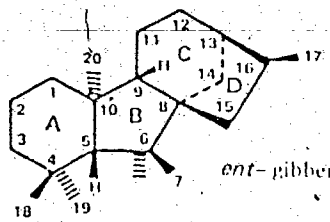
1.Extraction of GABPs

G-25 Eluate was prepared as described in Part C 'In Vitro Assay of GABPs'.

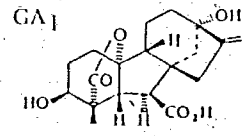
2.Gibberellins

Structures of the GAs and other compounds discussed in this chapter are shown in Figure D1. [^{3}H]GA_4 had been synthesized by NEN and was partially purified in our laboratory as described in Part C. [^{3}H]GA_1, prepared by catalytic reduction of GA_3, was also used in some experiments. Its purity and specific activity were similar to that of the NEN [^{3}H]GA_4. 3-Epi-GA_4 and C-7 methyl esters of GA_3, GA_4, and GA_7 were synthesized and purified by TLC. GA_4 and GA_7 were purchased from Abbott Laboratories, Chicago, IL; GA_6 was a gift from Dr. G. Sembdner, Institut für Biochemie der Pflanzen, Akademie der Wissenschaften, Saale, G.D.R.; 2,2 dimethyl GA_4 ($DiMeGA_4$) was a gift from Dr. R. Pharis, Dept. of Biology, University of Calgary, Alberta. The other GAs were generously donated by Dr. L. Rappaport, Plant Growth Laboratory, Dept. of Vegetable Crops, University of California, Davis. All GAs were stored in ethyl acetate, ethanol (1:1, v/v) at below -20 C. Some of the rare GAs were available in only minute quantities. Their purity and mass could not be

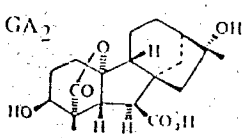
Figure D1. Structures of ent-gibberellane and of ligands used. (adapted from Bearder 1980)



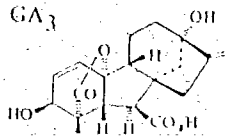
ent– gibberellane skeleton



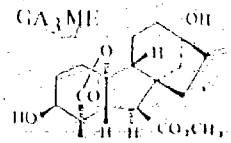
GA_1



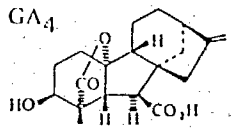
GA_2



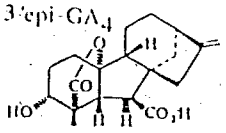
GA_3



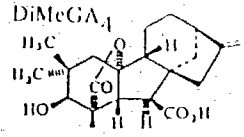
GA_3ME



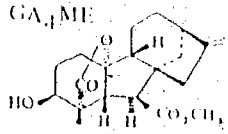
GA_4



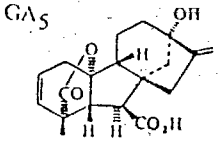
3-epi-GA_4



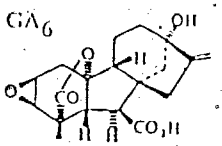
$DiMeGA_4$



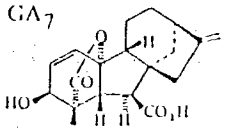
GA_4ME



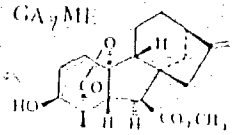
GA_5



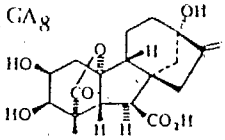
GA_6



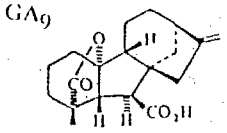
GA_7



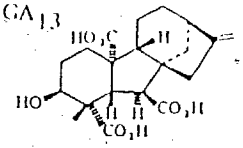
GA_7ME



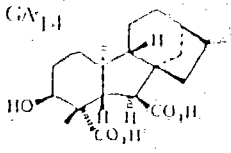
GA_8



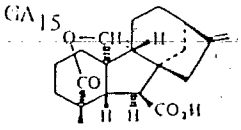
GA_9



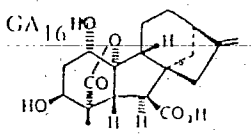
GA_{13}



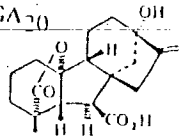
GA_{14}



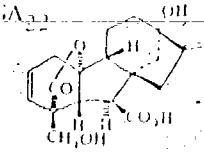
GA_{15}



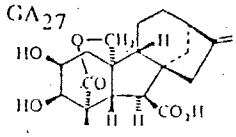
GA_{16}



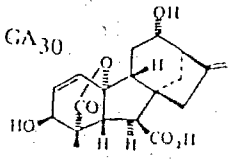
GA_{20}



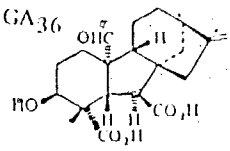
GA_{24}



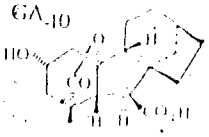
GA_{27}



GA_{30}

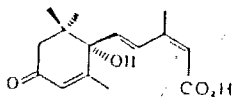


GA_{36}

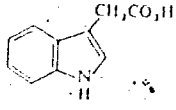


GA_{40}

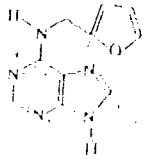
Abscisic acid



Indole-3-acetic acid



Kinetin



verified in our laboratory. Some of these were assayed only once, the other GAs and other hormones were used in two or more repeat experiments.

3. In vitro binding assays

DEAE cellulose filter assays for determination of bound radioactivity were performed as described in Section C, In Vitro Assay of GABPs.

Estimates of the affinity of the GABPs for the selection of different GAs and other substances were obtained by measuring the amount of [^{3}H]GA_4 binding displaceable by various concentrations of unlabeled competitor. In a typical experiment G-25 eluate was incubated with 50 nM [^{3}H]GA_4 in the absence or presence of competing ligand concentrations ranging from 50 nM to 0.5 mM, thereby giving an excess of competing ligand over [^{3}H]GA_4 of up to 10,000 times. In some instances, the competitor concentration ranged to 0.25 mM. In each experiment competitors were compared to unlabeled GA_4 as a standard. 100 µl samples containing 350 to 390 µg protein were used in the binding assay. Two or 3 replicates were used for each sample.

II. Results

The affinity of the GA-binding protein for various ligands was evaluated by studying the influence of their increasing concentration on the binding of [^{3}H]GA_4. Figure D2a illustrates the competition curves obtained using unlabeled GA_4 and GA_3 as competitors. Total binding is the amount of [^{3}H]GA_4 bound in the absence of any competitor. Nonspecific binding represents the amount of radioactivity bound in the presence of an excess of competitive ligand. Specific binding is the difference between total and nonspecific binding. It is noncovalent and exchangeable (see also Fig. D3). As the concentration of unlabeled ligand in the incubation mixture is increased, the number of exchangeably bound [^{3}H]GA_4 molecules is progressively reduced and the radioactivity adsorbed to the filter paper decreases. At 5 μM GA_4 the competition curve forms a plateau. At this point all those binding sites which reversibly bind [^{3}H]GA_4 are saturated by the excess of unlabeled GA_4. The amount of radioactivity still bound results from sites which bind [^{3}H]GA_4 irreversibly or with low affinity and is, therefore, nonspecific binding. The GA_3 displacement curve reaches the same plateau as that of GA_4. However, a much higher concentration of GA_3 is required to saturate all specific binding sites. The binding protein, therefore, has a lower affinity for GA_3 than for GA_4.

As a measure of the affinity of the binding protein for a ligand the I_{50} value for that ligand was determined. I_{50} is the

Figure D2. Displacement of [^{3}H]GA_4 binding from cucumber cytosol by GA_3 (a) and GA_{40} compared to GA_4 (b).
A 60% $(NH_4)_2SO_4$ pellet of cucumber hypocotyl cytosol was desalted. This fraction containing 3.6 mg soluble protein ml^{-1} was incubated with 50 nM [^{3}H]GA_4 in the absence or presence of competing ligands at the concentrations shown. I_{50} represents the concentration of competitor which displaces 50% of the exchangeable, specific binding of [^{3}H]GA_4 from the binding protein.

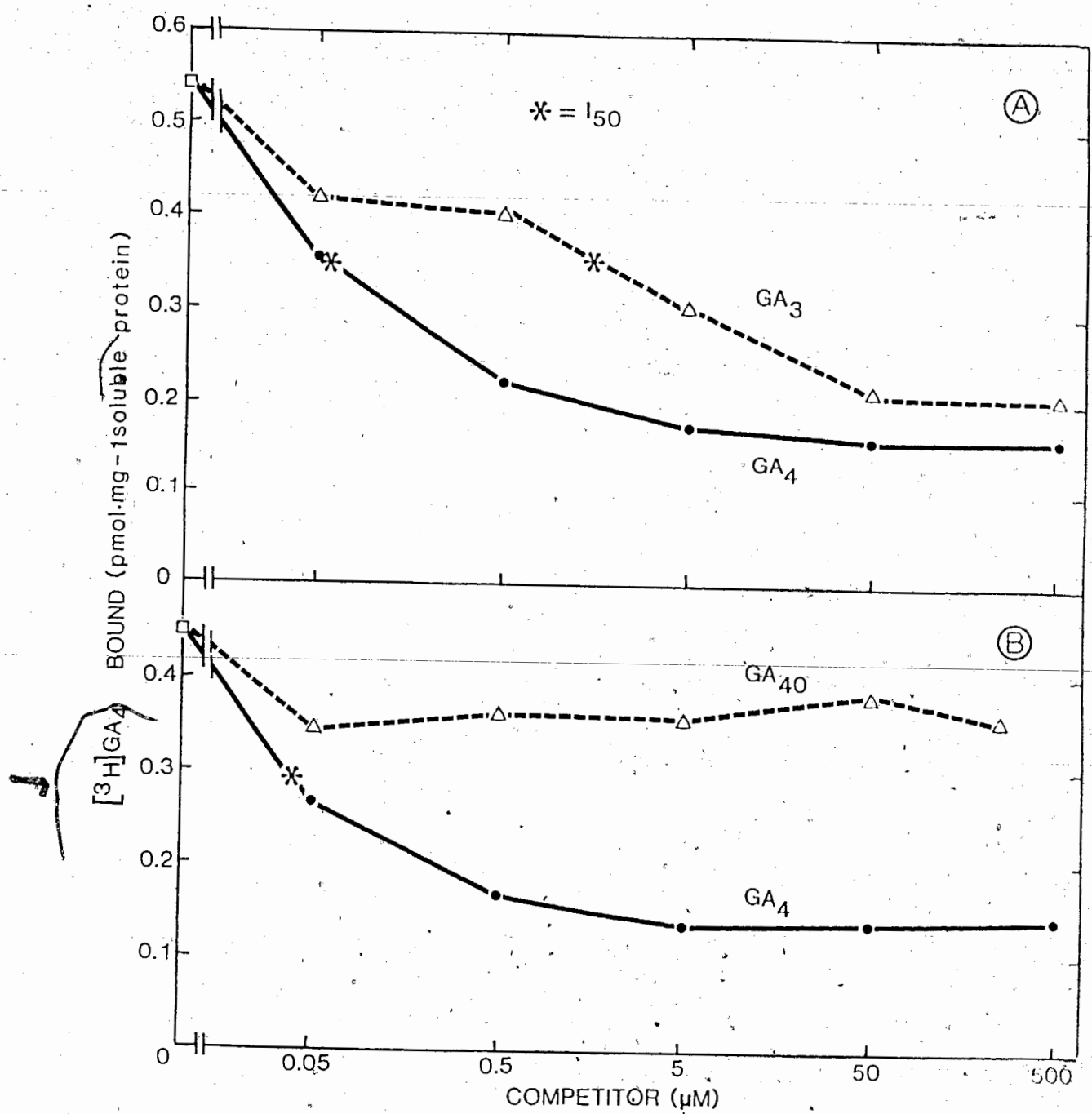
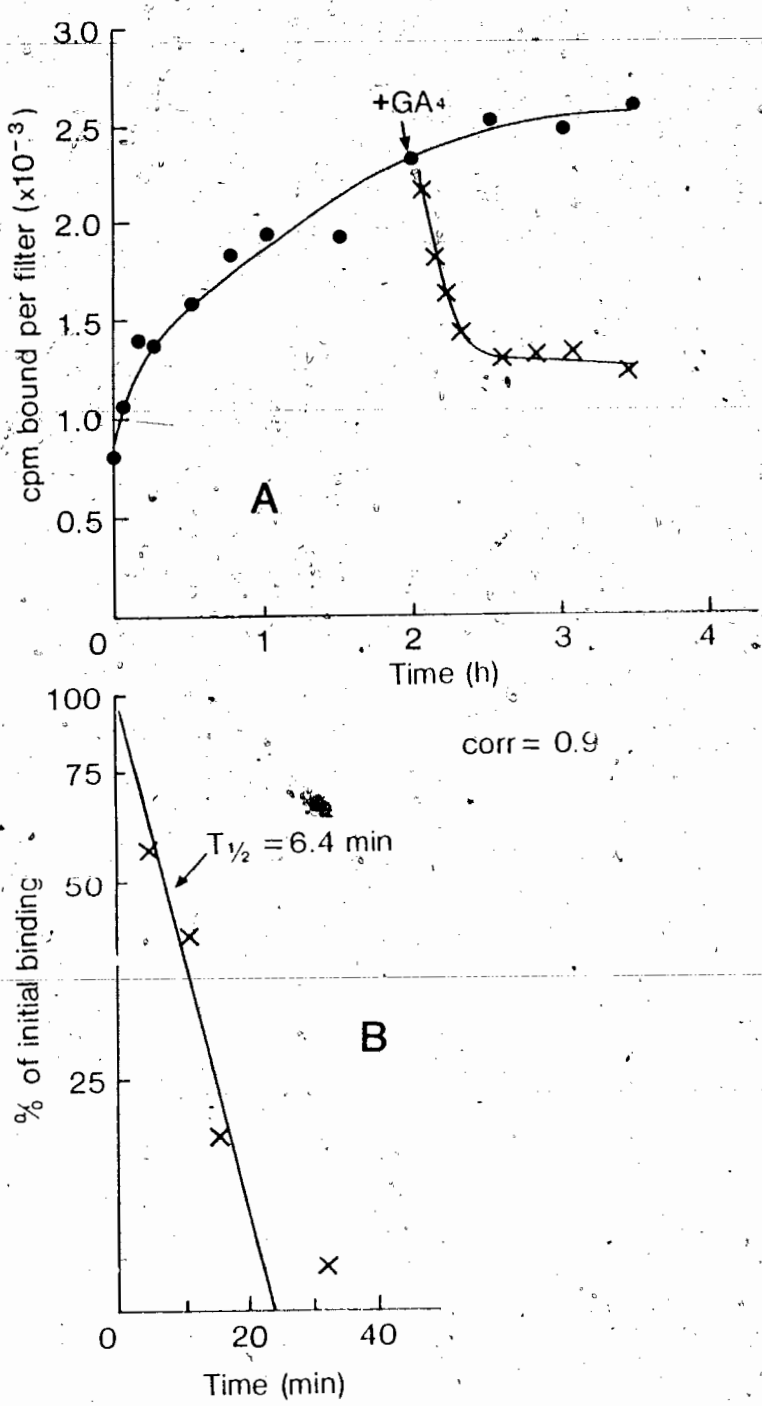

0.6
0.5
0.4
0.3
0.2
0.1
0
* = I50
A
GA3
GA4
B
GA40
GA4
BOUND (pmol·mg-1soluble protein)
[3H]GA4
0.05
0.5
5
50
500
COMPETITOR (μM)

Figure D3. Exchangeability of [^{3}H]GA_4 binding to G-25 eluate. G-25 eluate was incubated with 60 nM [^{3}H]GA_4. After 2 h a portion of the incubation mixture was added to a test tube containing 5 μM GA_4. Samples of both incubation mixtures were assayed at the times indicated (A). The data were used to determine the half life of dissociation (B).


3.0
2.5
2.0
1.5
1.0
0.5
cpm bound per filter (x10⁻³)
+GA4
A
0
1
2
3
4
Time (h)
100
75
50
25
% of initial binding
T½ = 6.4 min
corr = 0.9
B
0
20
40
Time (min)

concentration of a competitor that produces 50% displacement of exchangeably bound [^{3}H]GA_4. The I_{50} for GA_4 was about 50 nM whereas that for GA_3 in separate experiments was between 2.5 to 5.0 μM (Fig. D2a). Some ligands, such as GA_{40}, were unable to reach I_{50} at concentrations as high as 0.25 mM (Fig. D2b).

By determining the I_{50} value for a series of GAs, GA derivatives and other hormones, an affinity ranking of ligands for the [^{3}H]GA_4 binding protein was established (Table D1). Affinity was highest for γ-lactonic C-19 GAs with a 3 β-OH with or without two methyl groups at C-2 (GA_4, DiMeGA_4, GA_7). There were no noticeable differences between the I_{50} values for these ligands. Additional hydroxylation of C-16 in the D-ring (GA_2), of C-13 (GA_1, GA_3), and of C-12 (GA_{30}) in the C-ring progressively impeded binding. Changes in the hydroxylation pattern of the A-ring either curtailed binding affinity or completely eliminated it. Thus GAs with a 2,3 epoxide (GA_6), a 1 α-OH (GA_{16}), or a 2 β-OH (GA_8) were unable to displace exchangeable [^{3}H]GA_4 binding. Lack of a 3 β-OH (e.g., GA_5 and GA_9) resulted in greatly decreased affinity. If this lack was coupled to the addition of a 2 α-OH (GA_{40}), or the hydroxylation of C-13 (GA_{20}) and at C-18 (GA_{22}), binding affinity was lost.

The structural specificity of the binding protein is further illustrated by 3-epi-GA_4 which with a 3 α-OH showed a 10,000-fold lower affinity than GA_4 with a 3 β-OH. The slight [^{3}H]GA_4 displacement observed could be a result of the presence of 0.7% GA_4 as a contaminant in the 3-epi-GA_4, as determined by

Table D1. Relative *in vitro* affinities of various ligands to [^{3}H]GA_4 binding protein and their relative *in vivo* biological activity in the cucumber hypocotyl bioassay. Concentration of [^{3}H]GA_4 in the incubation mixture was 50 nM and that of competing ligands ranged from 50 nM to 0.5 mM, except for GAs with superscript[c]. Relative affinity was measured using I_{50} values determined for each ligand using the DEAE filter paper assay.

I_{50}	competitor
50 nM	GA_4 (+++),[a] $DiMeGA_4$ (+++),[b] GA_7 (++++)
0.5 μM	GA_2 (++)
5.0 μM	GA_1 (++), GA_3 (++)
50 μM	GA_5 (+), GA_9 (+++), GA_{30}[c] (+)
0.5 mM	3-epi-GA_4 (0)[d]
>0.5 mM	GA_3ME (0), GA_4ME (0), GA_6[c] (+), GA_7ME (0), GA_8 (0), GA_{13} (0), GA_{14} (0), GA_{15} (++), GA_{16}[c] (+), GA_{20} (0), GA_{22} (0), GA_{27} (0), GA_{36}[c] (+++), GA_{40}[c] (++), ABA (0), IAA (0), kinetin (0)

[a]Relative activity in the cucumber hypocotyl bioassay (++++, very high; +++, high; ++, moderate; +, low; 0, very low or inactive) after Crozier and Durley (1983), except for those with superscript[b] or [d].
[b]Relative *in vivo* activity after Hoad *et al.* (1981). [c]Competitor assayed *in vitro* at concentrations ranging from 50 nM to 0.25 mM.
[d]N. Yalpani and L.M. Srivastava (unpublished data).

GLC.

C-7 methyl esters of GA_3, GA_4 and GA_7, and C-20 GAs with a δ-lactone ring (GA_{15}, GA_{27}) or without a lactone ring (GA_{13}, GA_{14}, GA_{36}) showed no binding affinity. Other hormones such as ABA, IAA and kinetin also showed no binding affinity.

In reciprocal experiments using [^{3}H]GA_1 and unlabelled GA_1 or GA_4 as competing ligands, fraction containing the binding protein again showed a greater affinity for GA_4 than for GA_1. GA_3ME did not displace the exchangeable [^{3}H]GA_1 (Table D2).

Table D2. Effect of unlabeled competitors on displacement of $[^3H]GA_4$ and $[^3H]GA_1$ from GABP.
Data represent dpm bound·ml^{-1}.

Sample	Column buffer	G-25[a] eluate	% displacement
58 nM $[^3H]GA_4$	7,100	33,400	-
+ 5 μM GA_4	4,000	18,400	45
+ 5 μM GA_1	-	24,600	26
+ 50 μM GA_3ME	-	29,100	13
40 nM $[^3H]GA_1$	1,600	5,800	-
+ 5 μM GA_4	1,300	3,100	47
+ 5 μM GA_1	-	4,000	31
+ 50 μM GA_3ME	-	5,100	12

[a]G-25 eluate was assayed at 4.2 mg protein·ml^{-1}

III. Discussion

The GA structure--binding affinity data point to the presence of strong hydrophobic environments in the active site of the receptor protein corresponding to the 1 α, 2 α and β, 3 α, positions of the C-19 γ-lactonic GAs. They also suggest strong polar or ionic interactions in the vicinity of the 3 β-OH, the γ-lactone ring, and the C-6 carboxyl. Weak hydrophobic regions are likely present near C-12, C-13 and C-16.

My conclusions about the active site of the GA receptor in cucumber agree with those obtained by Serebryakov et al. (1984), who based theirs on results obtained from in vivo assays using selectively modified GAs (see Fig. B1). However, my results differ from theirs in the following aspects. While their data suggest obligatory hydrophobic interactions in the vicinity of C-11, C-12 and C-13, my results indicate that polar groups at these positions on the ligand reduce affinity but do not prevent binding. Furthermore, I propose the presence of a nonpolar region corresponding to the α plane of C-1, C-2, and C-3 which those authors did not examine.

My in vitro data on the relative affinities of GAs and GA derivatives for the [^{3}H]GA$_4$ binding protein generally support the activity rankings of the ligands in the cucumber hypocotyl bioassay. At the same time, they point to some refinements and important exceptions.

Thus, GA_1 and GA_3 both with C-3 and C-13 OH are reported to have lower activities than GA_4 or GA_7 with only a C-3 OH (Crozier and Durley 1983). My binding data indicate that the $[^3H]GA_4$ binding protein has an approximately 50 to 100 times lower affinity for GA_1 and GA_3 than for GA_4. GA_8, with a 2 β-OH, and C-7 methyl esters of GA_3, GA_4 and GA_7 are reported to have no activity *in vivo*. My binding data likewise indicate that 2 β-hydroxylation as well as methylation of the C-6 carboxyl lead to a complete loss of binding affinity. GA_7 is reported to have slightly higher activity than GA_4 and $DiMeGA_4$ in the cucumber bioassay (Hoad *et al.* 1981), yet my *in vitro* data show the same I_{50} value for these GAs.

The important exceptions are GA_9 and GA_{36}. GA_9, C-19 γ-lactonic but unhydroxylated GA, and GA_{36}, a C-20 3 β-hydroxylated GA, are both reported to have as high *in vivo* activity in the cucumber bioassay as GA_4 (Crozier and Durley 1983). They are believed to act as immediate biosynthetic precursors of GA_4 in cell free extracts and shoots of cucurbits (Hedden 1983, Graebe *et al.* 1980, Yamane 1987). My data show that GA_9 has very low and GA_{36} no affinity for the $[^3H]GA_4$ binding protein *in vitro*. Possibly GA_9 and GA_{36} have high activities *in vivo* because they are metabolized to the active GA in cucumber, presumably GA_4.

GA_{15}, a C-20 δ-lactonic GA, is also reported to have moderate activity in the cucumber hypocotyl bioassay (Crozier and Durley 1983). My data indicate, however, that the $[^3H]GA_4$

binding protein in cucumber has no affinity for GA_{15}. The moderate *in vivo* activity of GA_{15} may be due to the possibility that in solution it occurs in equilibrium with its C-20 alcohol open lactone form which is reported to be a precursor of GA_4 (Hedden 1983).

The observed structural specificity of the $[^3H]GA_4$ binding protein is common for proteins but the fact that some of the precursors of GA_4 biosynthesis in cucumber, namely GA_9 and GA_{36}, have little or no affinity for the binding protein suggests that we are dealing with a receptor protein rather than an enzyme of GA metabolism. An involvement of enzymes, such as glycosylases (Schneider 1983) in the observed binding of $[^3H]GA_4$ has, however, not been excluded.

The *in vitro* assay of the displacement of radioactive GA binding to cucumber cytosol under conditions of minimal GA metabolism is a powerful technique to determine those parts of the GA molecule which are important for a structural fit to a putative receptor. However, the technique is limited by the purity of the radiolabeled GAs and competing ligands and an accurate determination of their mass. Also, to exploit the technique to its fullest potential, the proportion of specific to nonspecific binding, which in turn is determined by the purity of the binding protein, should be as high as possible. In my assays, specific binding was routinely 50% or more of the total binding and, as mentioned earlier, the $[^3H]GA_4$ was about 61% pure. Efforts were therefore made to increase the purity of

the radioactive GAs and of the binding protein.

PART E

Partial Purification of a GABP Fraction.

The results from Parts C and D support the view that the GABP from cucumber extracts is a GA receptor. However, to test this hypothesis and identify the cellular function of the GABP, pure preparations of this protein are required. Efforts were therefore made to purify the GABP from cucumber extracts. For this purpose, salt fractionation, gel permeation, ion exchange and hydroxylapatite chromatography were examined. A number of other separation techniques were also tested.

I. Materials and Methods

1. Chemicals and materials

The source and purity of the GAs used has already been described in Part C, Materials and Methods.

Sephadex G-25 SF, G-75 SF, DEAE Sephadex A-50, and Sephacryl S 200 were purchased from Pharmacia. DE32 and DE51 anion exchange celluloses were products of Whatman. Hydroxylapatite - high resolution was bought from Calbiochem, and blue dextran-agarose (Matrix Gel Blue A) from Amicon. All chromatography materials were equilibrated and packed into columns as prescribed by the manufacturers. All other chemicals were of analytical grade.

2. Buffers

The following buffers were used:

A, 100 mM Tris, 1 mM EDTA, 5 mM dithiothreitol (DTT), 50 μM PMSF, adjusted to pH 7.3 with H_3PO_4;

B, 20 mM Tris, 1 mM EDTA, 5 mM DTT, adjusted to pH 7.0 with H_3PO_4;

C, 10 mM K-phosphate, 1 mM EDTA, 5 mM DTT, pH 7.0;

D, 200 mM K-phosphate, 1 mM EDTA, 5 mM DTT, pH 7.0;

E, 25 mM bis-Tris, adjusted to pH 6.3 with HCl;

F, 10% (v/v) Polybuffer 74 (Pharmacia), adjusted to pH 4.0 with

HCl;

G, 20 mM bis-Tris, 5 mM DTT, adjusted to pH 6.0 with HCl;

H, 20 mM piperazine, 5 mM DTT, adjusted to pH 5.5 with HCl;

I, buffer H adjusted to pH 5.0.

3. GABP extraction and purification

Hypocotyls were harvested into buffer A from seedlings grown as described in Part C. For large scale extractions exceeding 200 g of hypocotyl tissue, sections were homogenized with 5 bursts of 4 sec each in a Waring blender in an equal volume (1:1, w/v) of buffer A, otherwise a mortar and pestle were used. The homogenate was filtered through 2 layers of nylon screening and the filtrate centrifuged at 100,000g, 1.5 h, to yield the cytosol. $(NH_4)_2SO_4$ was added to the cytosol as described in 'Results'. After equilibration and centrifugation at 23,000g, 20 min, the pellet was washed, resuspended in buffer B and particulates removed at 7,000g, 5 min. The supernatant was desalted on a Sephadex G-25 SF column (2.5 x 16 cm). In early experiments the G-25 eluate was stored as a lyophilized powder at -30 C. Later it was stored at -70 C after freezing in liquid N_2.

Pooled G-25 fractions were loaded onto a DEAE-ion exchange column (DE32, Whatman) equilibrated in buffer B and eluted with a gradient of KCl as described in 'Results'. The GABP containing fraction was precipitated with 70% $(NH_4)_2SO_4$ and resuspended in

buffer B.

A number of approaches were tested for increasing the purity of this DE32 eluate:

a. Hydroxylapatite chromatography.

The matrix consists of calcium phosphate crystals and acts similar to an ion exchanger but also tends to bind calcium binding proteins. DE32 eluate in buffer C was applied to a 2.5 cm x 15 cm column and unbound protein washed out. GABP was eluted with a single step gradient of buffer D. The eluate was precipitated with 70% $(NH_4)_2SO_4$ and resuspended in a small volume of buffer B. It could be stored at -70 C after freezing in liquid N_2.

b. Dye-ligand chromatography

One-half ml of DE-32 eluate (containing 2.4 mg protein) in buffer B was allowed to adsorb for 30 min to a blue dextran-agarose column (1 cm x 3 cm). The matrix used has a tendency to bind dehydrogenases and kinases, as well as some other proteins (Anon. 1979). Unbound protein was washed out with 10 ml buffer B. The bound GABP was eluted with 10 ml 1.5 M KCl in buffer B. The eluates from several such separations were combined, precipitated with $(NH_4)_2SO_4$ and resuspended in buffer B for assay.

c. High performance liquid chromatography

Gel permeation chromatography was performed using a Waters chromatograph with 2 M510 pumps, a M680 gradient controller, and a M740 recorder/integrator at room temperature. I 125 and 300 SW (Waters) gel filtration columns connected in series were used. Samples (100 µl) were eluted with buffer D at 0.5 ml·min^{-1}. Blue dextran, ferritin, catalase, alcohol dehydrogenase, BSA, ovalbumin, and cytochrome C were used for molecular weight calibration.

d. Fast protein liquid chromatography

For chromatofocusing and anion exchange chromatography a Pharmacia FPLC chromatograph with 2 P500 pumps and a LCC500 gradient controller were used at 3 C.

Chromatofocusing. A Mono P HR 5/20 column (Pharmacia) was equilibrated with buffer E. Immediately prior to sample loading a 3 ml aliquot of buffer F was passed through the column. DE32 eluate was equilibrated with buffer E using a small desalting column. Four ml of eluate containing 6 to 8 mg protein were applied to the column at 0.3 ml·min^{-1}. After elution of unbound proteins, buffer F was passed through the column at 0.5 ml·min^{-1}. This resulted in a pH gradient from pH 7 to 4. Fractions were pooled for assay.

Anion exchange chromatography. Samples were chromatographed at 3 C on a prepacked Mono Q HR 5/5 (quaternary amino ethyl) ion

exchange column (Pharmacia). Chromatography was performed at pH 7.0 with buffer B, at pH 6 with buffer G, and at pH 5.5 and 5.0 with buffers H and I, respectively. Bound proteins were eluted with up to 1 M KCl in these buffers.

4. In vitro GABP assays

As was shown in part C, many macromolecules bind GAs resulting in apparent specific binding. In order to discriminate between this and receptor-type specific binding, each fraction was assayed with [^{3}H]GA_4 in the absence and presence of a 100 fold excess of unlabeled GA_4 (high in vivo activity), GA_3 (moderate in vivo activity), and a 1000 fold excess of GA_4ME (no in vivo activity). The resulting displacement of [^{3}H]GA_4 from the binding sites by the competitors was used as a guide to select those fractions containing the putative receptor. In these fractions the extent of [^{3}H]GA_4 displacement is expected to correlate with in vivo activity of the competitor. For most experiments 50 nM [^{3}H]GA_4 prepared from NEN reaction products was used. However in later specified experiments, 10 nM [^{3}H]GA_4 purchased from Amersham was used. At this lower concentration, background binding was reduced and the GABP could be detected at sample protein concentrations as low as 0.2 mg protein·ml^{-1}, thereby allowing increased assay sensitivity.

Triplicate 100 µl samples of incubation mixtures were assayed with the DEAE cellulose filter assay as described in

Part C.

5. Sodium dodecyl sulfate (SDS) PAGE

Samples were desalted using a small Sephadex G-25 column. The protein fraction was precipitated at -20 C for 1 h with 9 volumes of 10 mM β-mercaptoethanol in acetone. Precipitates were pelleted at 28,000g, 5 min, washed and then washed twice with anhydrous ether. The pellets were resuspended in Laemmli's sample loading buffer (Laemmli 1970) and boiled for 5 min. Electrophoresis was performed with 12 mA constant current at 2 C using a commercial slab gel apparatus (model SE 600, Hoefer Scientific Instruments, San Francisco CA). One and a half mm thick gels (separating gel: 8.5%T, 2.7%C; stacking gel: 4%T, 2.7%C) were used. Gels were silver stained as described by Wray et al. (1981).

II. Results

1. Source of tissue extract

Specific binding was observed in G-25 eluate prepared from dry cucumber seeds as well as seeds germinated in running water for up to 96 h (Table E1). However, on a mg protein and a fresh weight basis, hypocotyls of 7 day old seedlings showed greater amounts of ectractable specific binding (Figs. E1 and E2). Furthermore, target (apical) as well as nontarget (basal) regions of the hypocotyls of 7 day old seedlings bound GA_4 specifically (Fig. E2). On a per mg protein basis both the apical and basal regions had a similar number of binding sites ($n = 0.1$ pmol·mg^{-1} protein) and affinity ($K_d = 30$ nM) for GA_4. However on a fresh weight basis, 6 times more GA binding sites were observed in the apical tissues ($n = 0.2$ pmol·g^{-1} fresh weight) compared to the nontarget region ($n = 0.03$ pmol·g^{-1} fresh weight).

Although harvesting apical hypocotyl sections from 7 day old seedlings is very labor intensive, this material was chosen for GABP purification. It was expected that purification of this protein would be facilitated by using source tissue which is enriched in this protein, or by excluding tissues which have a high concentration of other proteins.

Table E1. [^{3}H]GA_4 binding to G-25 eluate from whole seeds germinated for various periods of time and from apical parts of hypocotyls from 7 day old seedlings. Seeds were germinated in running water for the duration shown. Whole seeds were extracted in the same manner as 7 day old hypocotyl sections. Data represent pmol [^{3}H]GA_4 (Amersham) bound·mg^{-1} protein. All samples were assayed at a protein concentration of 2 mg·ml^{-1}.

Incubation	seed germination time						hypocotyls
	0 h	8 h	16 h	24 h	48 h	96 h	7 days
10 nM [^{3}H]GA_4	0.17	0.18	0.15	0.21	0.15	0.17	0.29
+ 1 μM GA_4	0.06	0.07	0.05	0.11	0.06	0.09	0.09
+ 1 μM GA_3	0.12	0.13	0.12	0.15	0.12	0.16	0.21
+10 μM GA_4ME	0.14	0.15	0.14	0.19	0.16	0.16	0.27

Figure E1. Scatchard plot of specific binding of GA_4 to G-25 eluate from ungerminated seeds.

Amersham [^{3}H]GA_4 was used. The data point at '*' suggests the presence of a second class of binding sites with a low K_d.

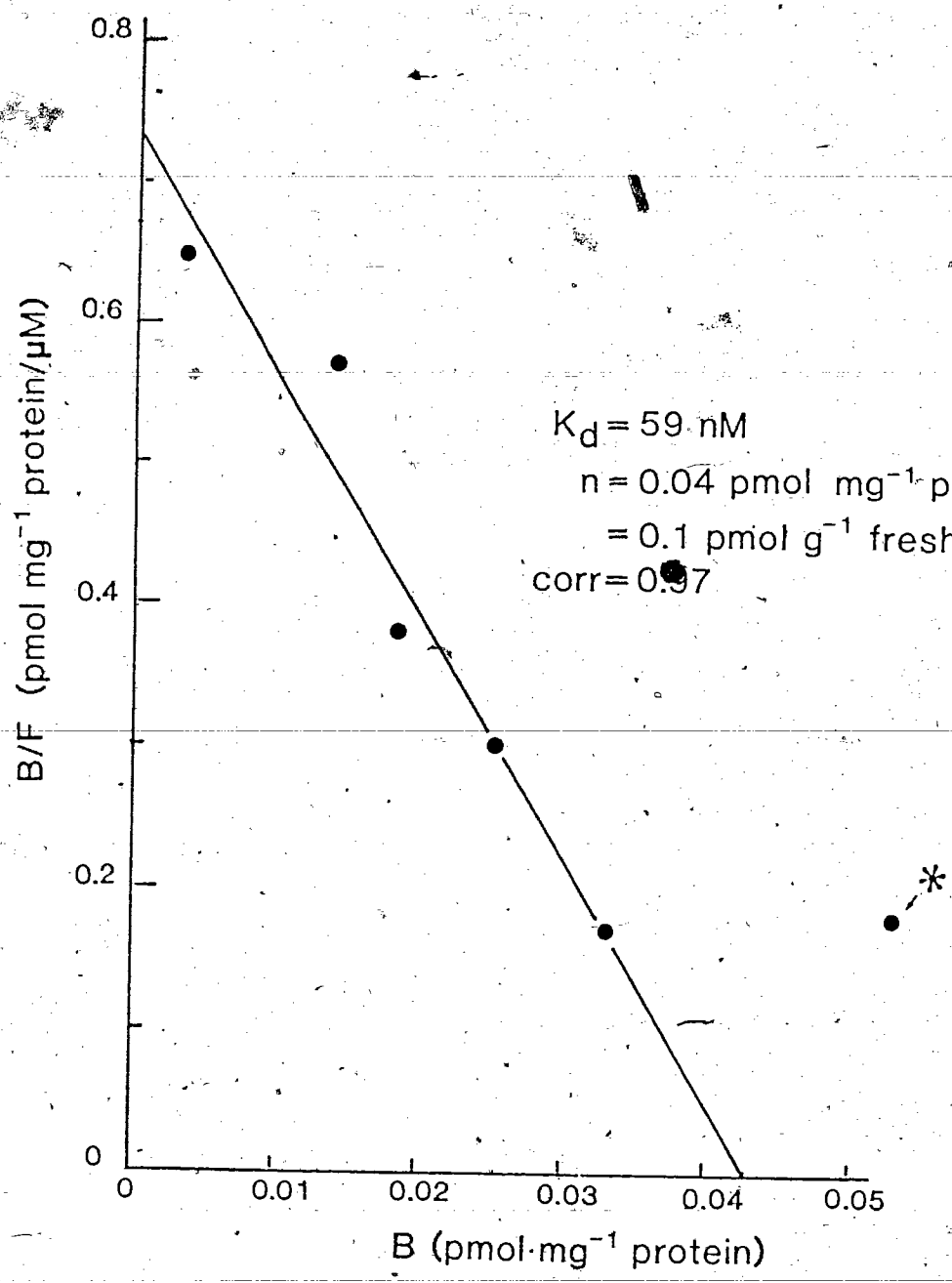
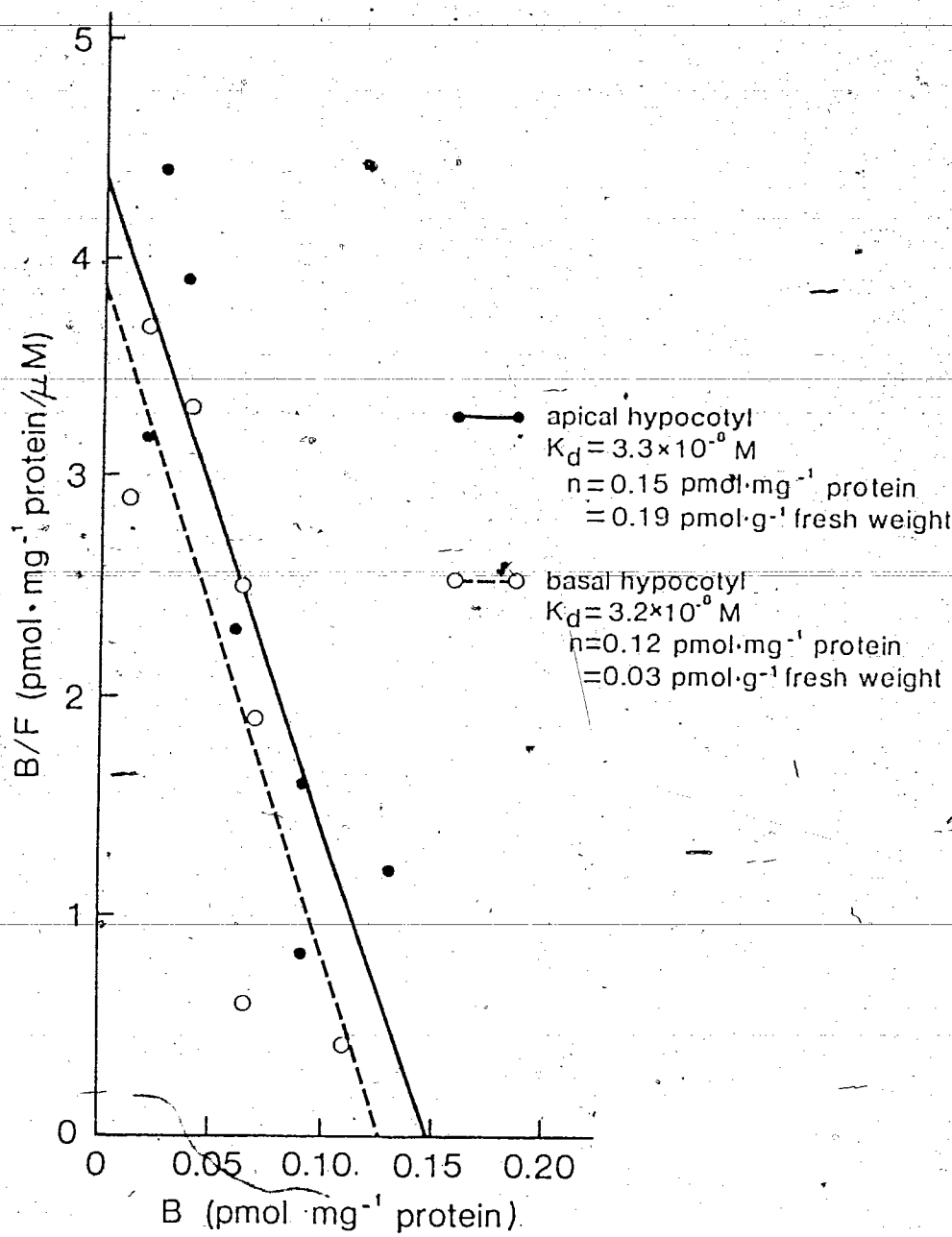

0.8
0.6
0.4
0.2
0
B/F (pmol mg^{-1} protein/μM)
K_d = 59 nM
n = 0.04 pmol mg^{-1} protein
= 0.1 pmol g^{-1} fresh weight
corr = 0.97
0
0.01
0.02
0.03
0.04
0.05
B (pmol·mg^{-1} protein)

Figure E2. Scatchard plot of specific binding of GA_4 to cytosol from apical and basal hypocotyls of 7 day old seedlings.


5
4
3
2
1
0
0
0.05
0.10
0.15
0.20
B (pmol mg⁻¹ protein)
B/F (pmol·mg⁻¹ protein/μM)
apical hypocotyl
Kd = 3.3×10⁻⁶ M
n = 0.15 pmol·mg⁻¹ protein
= 0.19 pmol·g⁻¹ fresh weight
basal hypocotyl
Kd = 3.2×10⁻⁶ M
n = 0.12 pmol·mg⁻¹ protein
= 0.03 pmol·g⁻¹ fresh weight

2. Effect of dithiothreitol

Dithiothreitol (DTT) is a reagent for the protection of sulfhydryl groups of proteins (Cleland 1964). Its effects on levels and stability of receptor-type binding were tested. The results shown in Tables E2 and E3 indicate that the presence of DTT throughout the GABP extraction significantly enhances specific binding before and after storage at -70 C. The effect is reversible as its removal from the extract results in a loss of this protection. Addition of DTT to a GABP extract, which had been partially purified in its absence, resulted in a slight enhancement of receptor-type binding. Five mM DTT offered the same protection as 10 mM DTT. All purification buffers therefore included 5 mM DTT.

3. Ammonium sulfate precipitation

Differences in the charge characteristics of proteins affecting their solubility in $(NH_4)_2SO_4$ solutions were exploited for the concentration and partial purification of the GABP from cytosol. The experiments in the previous chapters were performed using G-25 eluate prepared from cytosol precipitated with 60% $(NH_4)_2SO_4$. It was attempted to refine this step further with the next experiment.

The 100,000g cytosol of hypocotyl extracts was divided into 5 portions. $(NH_4)_2SO_4$ was added to each portion to yield

Table E2. Effect of presence of DTT on GA binding to G-25 eluate.
G-25 eluate from hypocotyls was prepared using extraction and column buffers lacking (no DTT) or containing 10 mM dithiothreitol (DTT throughout). In one extract DTT was removed from the $(NH_4)_2SO_4$ pellet in the desalting step (DTT removed). Data represent $[^3H]GA_4$ bound (pmol·mg^{-1} protein).

Incubation	no DTT	DTT throughout	DTT removed
50 nM $[^3H]GA_4$	0.10	0.16	0.09
+ 5 μM GA_4	0.04	0.05	0.05
+ 5 μM GA_3	0.07	0.09	0.07
+50 μM GA_4ME	0.09	0.14	0.10

Table E3. Effect of 10 mM DTT on stability of G-25 eluate stored at -70 C for 3 days.

G-25 eluate from extracts prepared as described in Table E13 was used for storage. 'DTT added' was prepared by adding DTT to 'no DTT' extract upon thawing. Data represent [^{3}H]GA_4 bound (pmol·mg^{-1} protein).

Incubation	no DTT	DTT throughout	DTT added
50 nM [^{3}H]GA_4	0.11	0.13	0.08
+ 5 μM GA_4	0.07	0.04	0.05
+ 5 μM GA_3	0.07	0.09	0.07
+50 μM GA_4ME	0.09	0.10	0.08

concentrations of 30, 40, 50, 60, and 85% saturation. The pellets obtained after centrifugation were desalted by gel filtration and assayed for specific binding. The results (Table E4) indicate that the GABP precipitates out at an $(NH_4)_2SO_4$ concentration of 40% or more. In another experiment, $(NH_4)_2SO_4$ was added to cytosol to sequentially reach 30, 40, 50, and 60% saturation. Pellets obtained at each step were removed for assay. As shown in Table E5, the putative receptor appears to precipitate out with 30 to 60% $(NH_4)_2SO_4$, with the majority precipitating with 50% $(NH_4)_2SO_4$. No GABP was detected in 60-80% $(NH_4)_2SO_4$ pellets (data not shown). Only 1 to 10% of the total cytosol proteins precipitate out with the 30% $(NH_4)_2SO_4$. For this reason, and to save time, cytosol was precipitated with 50% $(NH_4)_2SO_4$ in early experiments. In later experiments, however, cytosol was precipitated with 55% $(NH_4)_2SO_4$ after the 30% pellet was removed. This allowed increased yield and removal of some lipids and hydrophobic proteins which tend to adsorb to chromatography matrices in subsequent purification steps. Precipitating GABP over a narrower range of $(NH_4)_2SO_4$ concentations does not appear to be practical.

About 50% of the total cytosol proteins are removed from the preparation with this purification step.

Table E4. Binding of [^{3}H]GA_4 to cytosol fractions cut with $(NH_4)_2SO_4$.
Cytosol was divided into 5 aliquots. $(NH_4)_2SO_4$ was added to each fraction to give the saturation shown. Each pellet obtained was desalted prior to assay. Data represent [^{3}H]GA_4 bound (pmol·mg^{-1} protein).

Incubation	Buffer B	$(NH_4)_2SO_4$ Pellet				
		30%	40%	50%	60%	85%
50 nM [^{3}H]GA_4	0.31	0.95	0.31	0.30	0.30	0.34
+ 5 μM GA_4	0.13	0.37	0.18	0.19	0.22	0.21
+ 5 μM GA_3	0.13	0.37	0.23	0.20	0.21	0.23
+50 μM GA_4ME	0.12	0.38	0.30	0.30	0.29	0.32

Table E5. Binding of [^{3}H]GA_4 to cytosol fractions sequentially cut with $(NH_4)_2SO_4$.
Cytosol proteins were precipitated with 30% $(NH_4)_2SO_4$. The pellet obtained was retained for assay while the supernatant was further fractionated with increasing $(NH_4)_2SO_4$ saturation as shown. Each pellet obtained was desalted prior to assay. Data represent [^{3}H]GA_4 bound (pmol·mg^{-1} protein).

Incubation	Cytosol	$(NH_4)_2SO_4$ Pellet			
		30%	30-40%	40-50%	50-60%
50 nM [^{3}H]GA_4	0.38	0.63	0.68	0.62	0.44
+ 5 μM GA_4	0.22	0.25	0.34	0.28	0.34
+ 5 μM GA_3	0.24	0.27	0.30	0.46	0.32
+50 μM GA_4ME	0.32	0.37	0.40	0.50	0.38

4. Open column ion exchange chromatography

A number of ion exchange chromatography matrices were tested, including DEAE Sephacel, DEAE Sephadex A-50, DE32, and DE51. The DE32 ion exchange cellulose had the best flow properties and gave the best chromatographic separation of G-25 eluate. Two column sizes were used. For G-25 eluate containing up to 70 mg total protein a 2.5 x 13.5 cm column was used; for large-scale preparations containing up to 400 mg protein a 5.0 x 16.5 cm column provided optimal separations. Typical elution profiles for these columns are shown in Figure E3. When loading samples in buffer B, all receptor - type binding activity was retained in the column. GABP could be eluted with 0.23 to 0.30 M KCl using a linear gradient of 0.18 to 0.33 M KCl (Tables E6 and E7). About 18% of the total proteins loaded onto the column elute with the GABP fraction. This chromatographic step results in about a 4-fold enhancement of the specific binding detectable in the G-25 eluate (Table E8).

5. Further purification of DE32 eluate

a. Hydroxylapatite chromatography

A slight enhancement of specific binding was achieved by passing the active fraction from the DEAE cellulose through a hydroxylapatite (HyAp) column. Crystals of calcium phosphate form the matrix of this column. Differences in charge and calcium binding properties of proteins are exploited with this

Figure E3. Elution profiles of G-25 eluate on 2.5 x 13.5 cm (a) and 5.0 x 16.5 cm (b) DE32 anion exchange columns. Regions marked A to F were assayed for GA-binding activity. The fractions labeled A to C in (a) are not equivalent to those in (b).

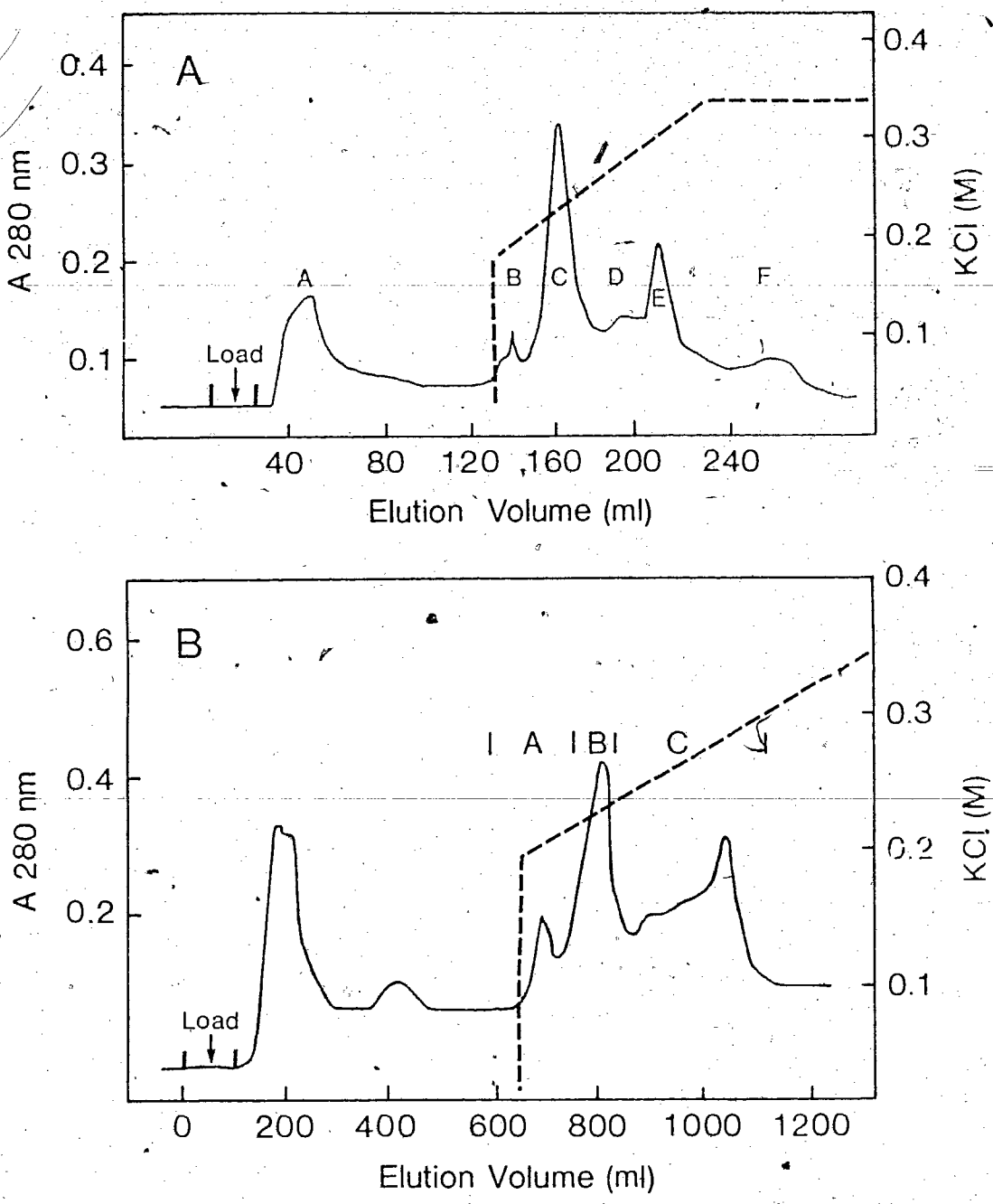

A
A 280 nm
KCl (M)
Load
A
B
C
D
E
F
40
80
120
160
200
240
Elution Volume (ml)
B
A 280 nm
KCl (M)
I A IBI C
Load
0
200
400
600
800
1000
1200
Elution Volume (ml)

Table E6. Binding of [^{3}H]GA_4 to fractions from a 2.5 x 13.5 cm DE32 anion exchange column.
Pooled, lyophilized fractions, collected according to Fig. E3a, were used for the incubations. Values given represent [^{3}H]GA_4 bound (pmol·mg^{-1} protein). All fractions were assayed at a protein concentration of 2.2 mg·ml^{-1}.

Fraction	A	B	C	D	E	F
50 nM [^{3}H]GA_4	0.30	0.13	0.17	0.27	0.38	0.09
+ 5 μM GA_4	0.20	0.09	0.08	0.11	0.17	0.13
+ 5 μM GA_3	0.20	0.09	0.10	0.15	0.21	0.05
+50 μM GA_4ME	0.19	0.09	0.11	0.22	0.30	0.08

Table E7. Binding of [^{3}H]GA_4 to cytosol, G-25 eluate, and fractions from a 5.0 x 16.5 cm DE32, and a 2.5 x 14.8 cm HyAp column.

DE32 fractions were collected as shown in Fig. E3b, frozen in liquid N_2 and stored at -70 C prior to assay. Values given represent [^{3}H]GA_4 bound (pmol·mg^{-1} protein). All fractions were assayed at a protein concentration of 2.9 mg·ml^{-1}, except cytosol (1.3 mg·ml^{-1}) and HyAp unbound (0.8 mg·ml^{-1}).

Fraction	cytosol	G-25 eluate	DE32 A	DE32 B	DE32 C[a]	HyAp unbound	HyAp bound
50 nM [^{3}H]GA_4	0.15	0.08	0.06	0.09	0.23	0.13	0.26
+ 5 μM GA_4	0.09	0.03	0.03	0.03	0.03	0.11	0.04
+ 5 μM GA_3	0.09	0.07	0.06	0.05	0.13	0.09	0.14
+50 μM GA_4ME	0.11	0.07	0.04	0.08	0.19	0.11	0.23

[a]Fraction C was loaded onto the hydroxylapatite column

Table E8. Purification of GABP from cucumber cytosol. Specific activity values calculated from data presented in Table E7. The difference between [^{3}H]GA_4 binding in absence and presence of GA_4 was used. Specific binding in cytosol is overestimated in these calculations because GA_4ME also displaces a large amount of radiolabel in this fraction (see Table E7). This results in a greater than 2-fold underestimation of the extent of purification and recovery achieved.

Fraction	total protein (mg)	specific activity (pmol per mg prot.)	total specific activity (pmol)	purification	recovery (%)
cytosol	872	0.06	52.32	1	100
G-25 eluate	384	0.05	19.20	1	37
DE32	72.4	0.20	14.48	3.3	28
HyAp bound	39.9	0.22	8.78	3.7	17

method (Gorbunoff and Timasheff 1984). As shown in Table E7, the GABP is bound by the HyAp if the samples are loaded in buffer F. The GABP can be eluted with a single step gradient using buffer G. This procedure results in only 10% improvement of specific binding of the DE32 eluate. About 10% of the proteins loaded onto the column are unbound and thereby are removed from the GABP.

Table E8 shows that with this procedure the GABP was purified about 3.5-fold, with about 17% recovery of the specific binding present in cytosol.

GA_4 binding to the HyAp fraction is saturable (Fig. E4). Scatchard analysis of the HyAp fraction suggests the presence of a single class of binding sites with 0.6 pmol binding sites per mg protein and a K_d near 50 nM (Fig. E5, compare with Fig. E2). The differences in K_d between cytosol and the HyAp extract may not be significant. They may also be related to the use of different sources of $[^3H]GA_4$ for the different samples.

The elution profile of the HyAp fraction from analytical runs on combined HPLC gel permeation columns is shown in Fig. E6b. Comparison of the chromatogram of the HyAp fraction with that of the DE32 eluate indicates that HyAp enhances the proportion of a high molecular weight (>400 kdalton) and a 110 kdalton protein (Fig. E6). Figure E7 shows the SDS-PAGE profile of this extract. Compared to G-25 eluate, the HyAp fraction again shows an enhanced band at 110 Kdalton. However, peptides

Figure E4. Saturability of GA_4 binding by the HyAp-bound fraction.
The HyAp-bound fraction was incubated with Amersham [^{3}H]GA_4 at the concentrations shown (Free). A 100-fold excess of GA_4 was used for deriving specific from nonspecific and total binding values. The extract was assayed at a protein concentration of 0.7 mg·ml^{-1}.

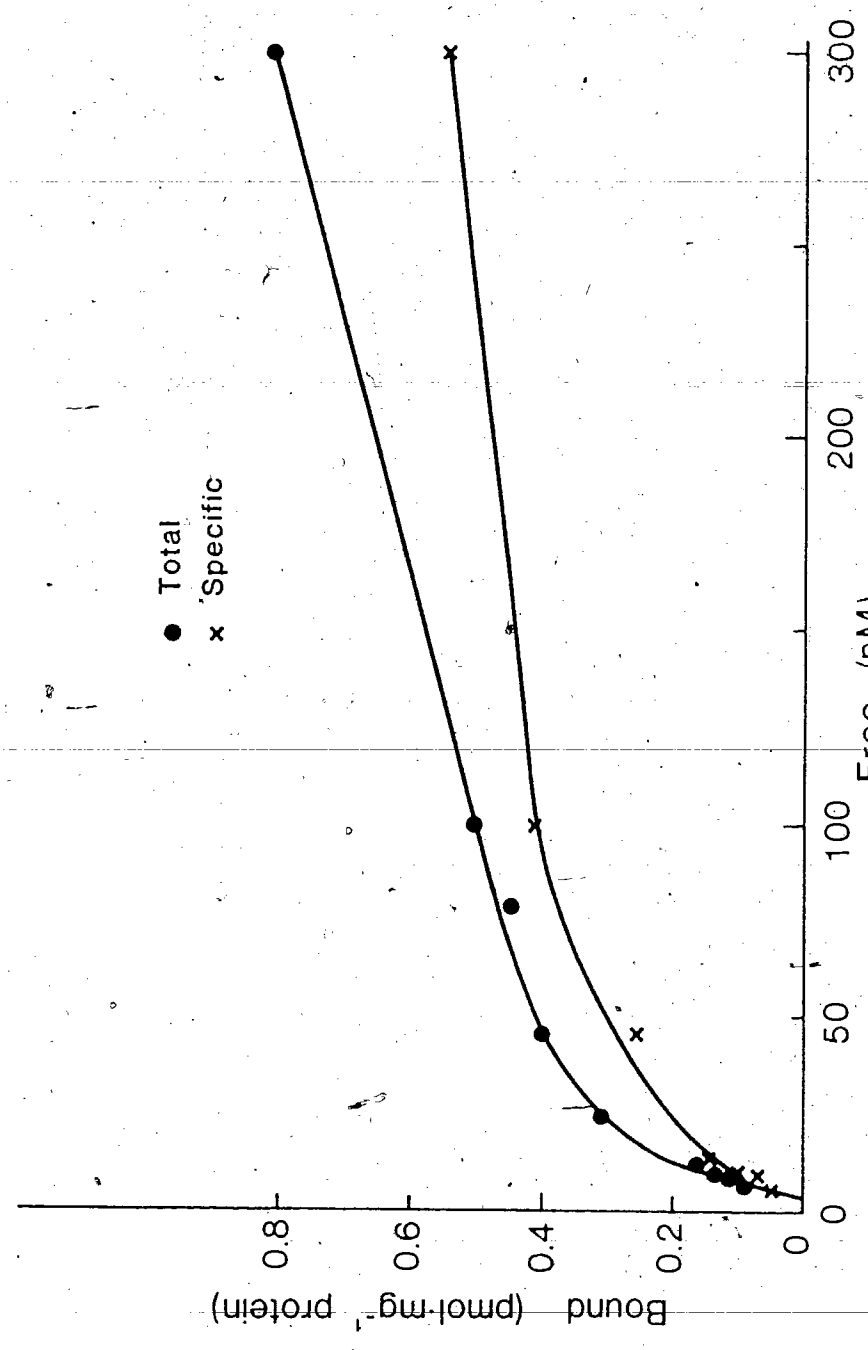

Bound (pmol·mg⁻¹ protein)
0.8
0.6
0.4
0.2
0
0
50
100
200
300
Free (nM)
Total
Specific

Figure E5. Scatchard analysis of HyAp-bound fraction. Amersham [^{3}H]GA_4 was used.

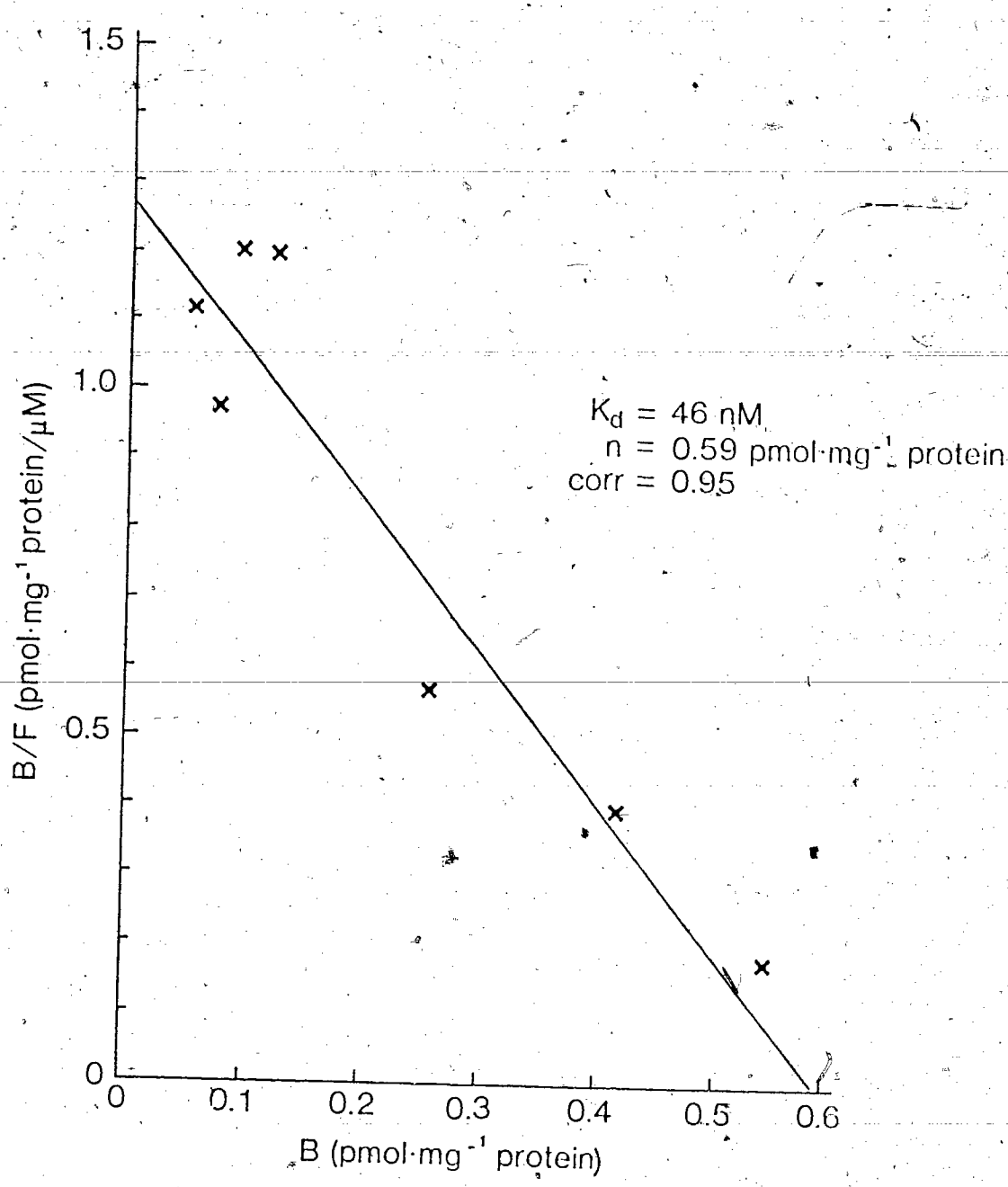

1.5
1.0
0.5
0
0
0.1
0.2
0.3
0.4
0.5
0.6
B/F (pmol·mg⁻¹ protein/μM)
B (pmol·mg⁻¹ protein)
K_d = 46 nM
n = 0.59 pmol·mg⁻¹ protein
corr = 0.95

Figure E6. Elution profile of DE32 eluate (a) and HyAp-bound (b) on HPLC gel filtration columns.

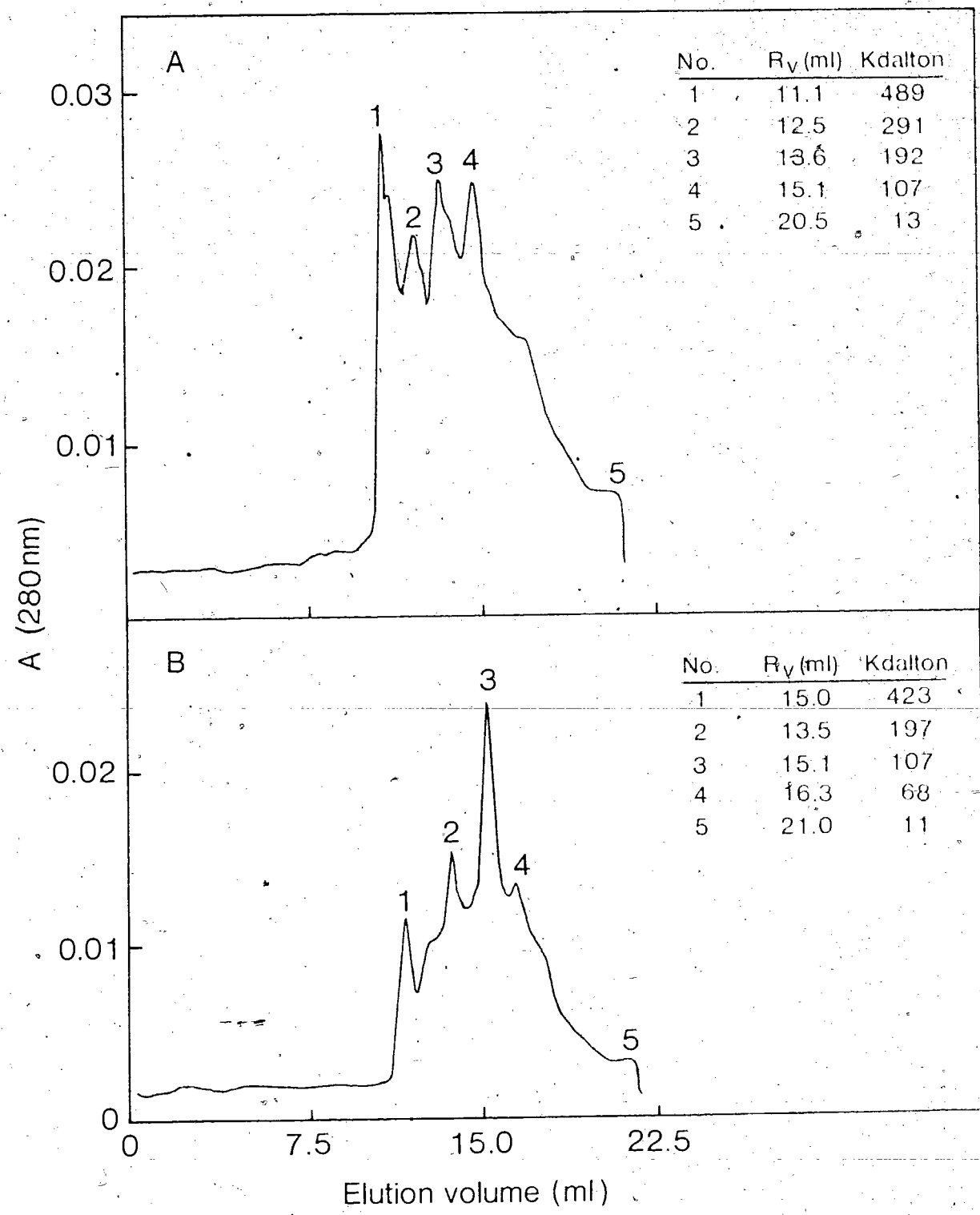
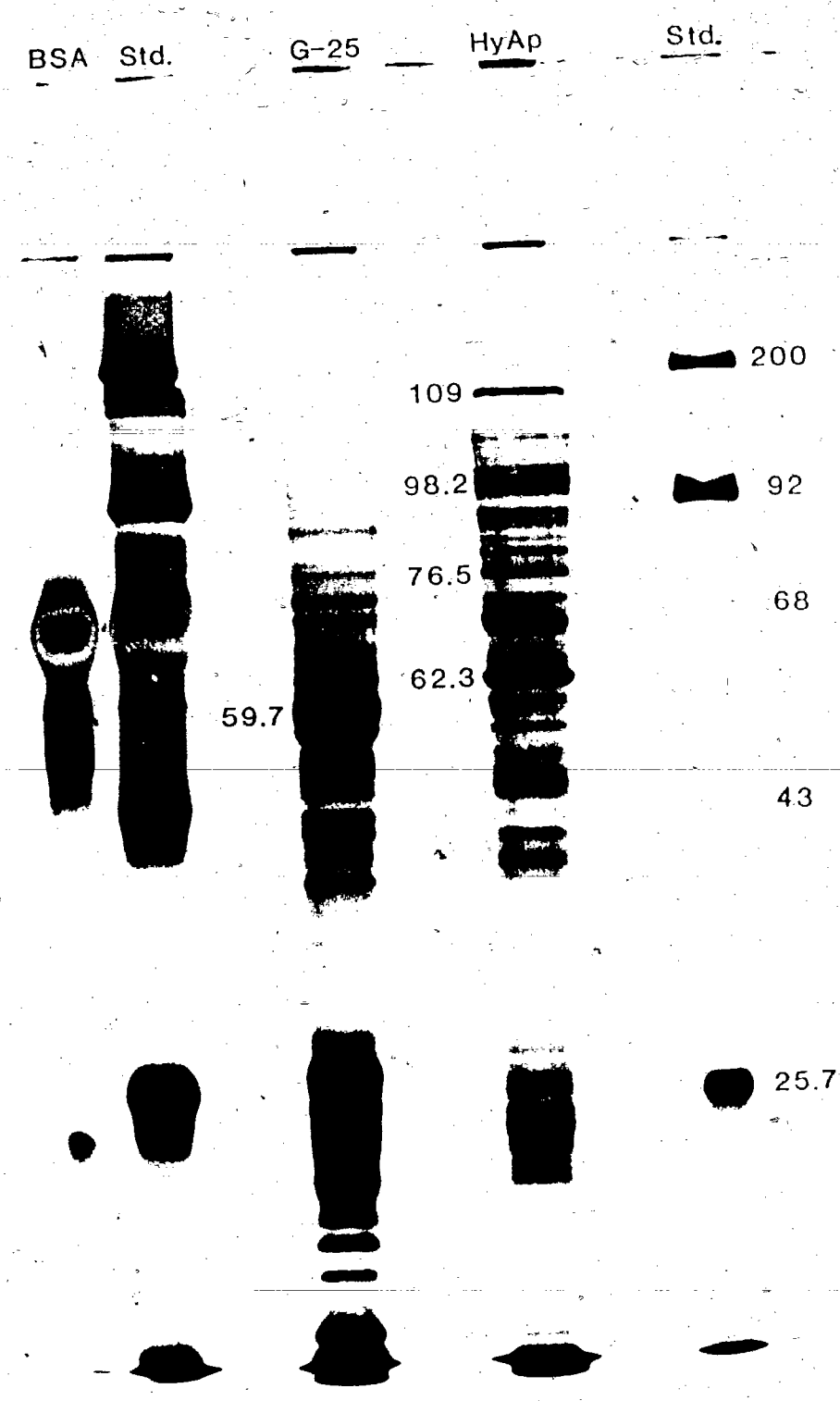

A
B
No. R_V (ml) Kdalton
1 11.1 489
2 12.5 291
3 13.6 192
4 15.1 107
5 20.5 13
No. R_V (ml) Kdalton
1 15.0 423
2 13.5 197
3 15.1 107
4 16.3 68
5 21.0 11
A (280nm)
0.03
0.02
0.01
0
0
7.5
15.0
22.5
Elution volume (ml)

Figure E7. SDS PAGE of G-25 eluate and HyAp-bound fraction. Molecular weights of some of the bands differing between these extracts are shown.


BSA
Std.
G-25
HyAp
Std.
200
109
98.2
92
76.5
68
62.3
59.7
43
25.7

with a molecular weight near 62, 76, and 98 Kdalton are also enhanced. Numerous other protein bands are still apparent in the HyAp fraction.

Problems with recovery and column performance have prevented successful further purification of the HyAp fraction.

b. Dye-ligand chromatography

An almost 3-fold purification of the DE32 eluate was achieved by chromatography on a Blue dextran-agarose column. As shown in Table E9, the GABP is bound by this matrix and can be eluted with 1.5 M KCl. However, only 24% of the specific binding and 23% of the total protein loaded was recovered from this column.

c. FPLC - Chromatofocusing

A Mono P chromatofocusing column was used on the FPLC to fractionate the DE32 eluate with a pH 6.5 to 4.0 gradient. On such a column proteins elute at a pH lower than their pI. This is due to their low solubility at their pI and Donnan potential effects near the chromatofocusing matrix -- buffer interface (Anon. 1982). A typical elution profile (Fig. E8) shows that a large proportion of the proteins in this extract elute between pH 5 and 4. The <u>in vitro</u> assay of eluate fractions (Table E10) indicates that the GABP also elutes with the majority of the proteins. Only a slight enhancement of specific binding was observed in this fraction compared to the starting material.

Table E9. [^{3}H]GA_4 binding to DE32 eluate and fractions unbound and bound by Blue dextran-agarose.
Fractions were assayed with Amersham [^{3}H]GA_4. Values represent [^{3}H]GA_4 bound (pmol·mg^{-1} protein).

Incubation	DE32	Blue A-agarose unbound	Blue A-agarose bound
10 nM [^{3}H]GA_4	0.13	0.07	0.33
+ 1 μM GA_4	0.06	0.06	0.12
+ 1 μM GA_3	0.08	0.06	0.17
+10 μM GA_4ME	0.10	0.06	0.27

Figure E8. Elution profile of DE32 eluate from a chromatofocusing column.
Fractions were collected for assay as indicated.

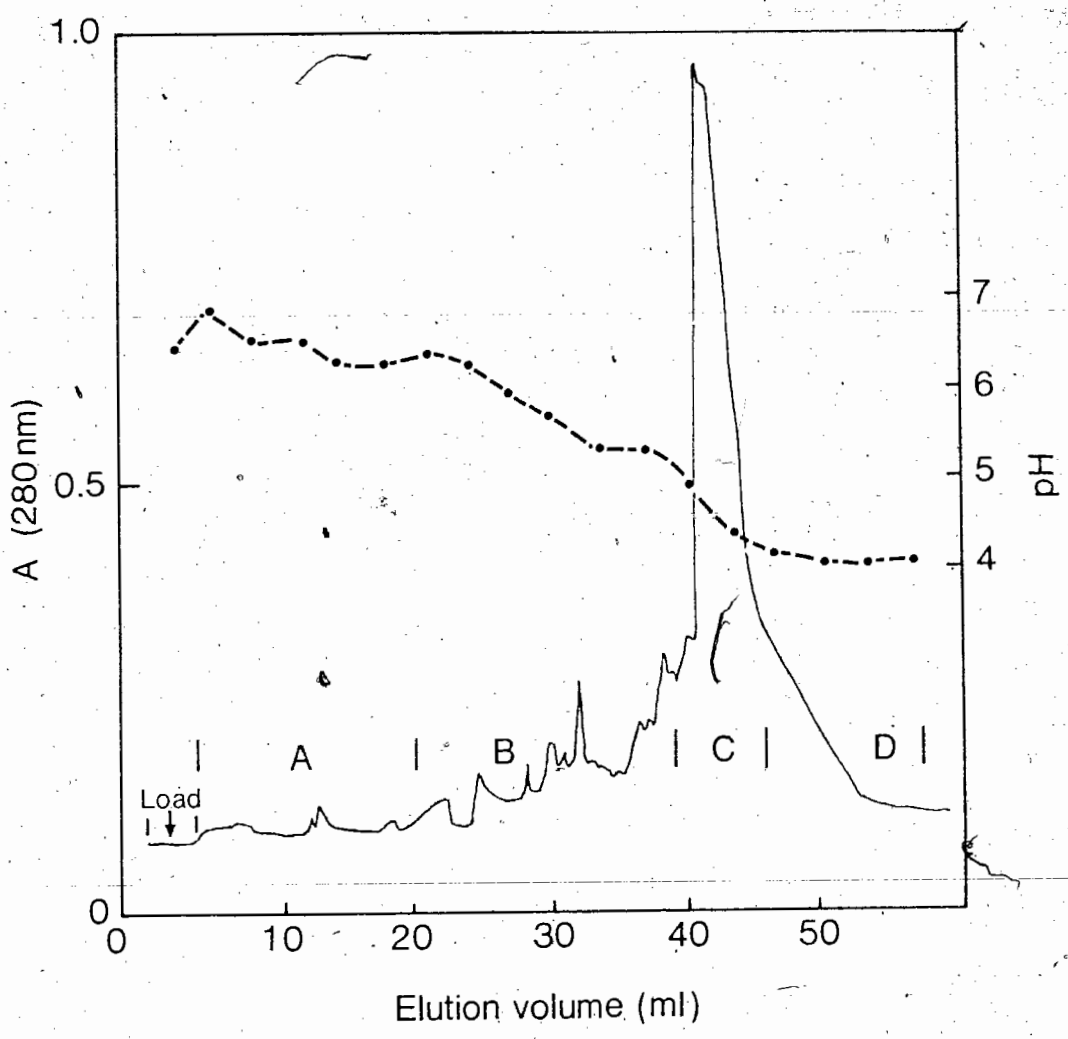

1.0
0.5
0
A (280 nm)
7
6
5
4
pH
Load
A
B
C
D
0
10
20
30
40
50
Elution volume (ml)

Table E10. [^{3}H]GA_4 binding to DE32 eluate and fractions eluted from chromatofocusing column (Mono P).
Fractions were collected according to Fig. E8 and assayed with Amersham [^{3}H]GA_4. Data represent [^{3}H]GA_4 bound (pmol·mg^{-1} protein).

Sample	DE32	Mono P eluate			
		A	B	C	D
(pH)	(7.0)	(6.9-6.3)	(6.3-5.1)	(5.1-4.1)	(4.1-4.0)
10 nM [^{3}H]GA_4	0.15	0.04	0.11	0.11	0.07
+ 1 μM GA_4	0.06	0.02	0.08	0.05	0.04
+ 1 μM GA_3	0.07	0.02	0.06	0.08	0.05
+10 μM GA_4ME	0.09	0.02	0.06	0.10	0.04

Only 20 to 35% of the total protein loaded onto this column could be recovered for assay. This method, therefore, did not appear to be practical for the preparative purification of the GABP.

d. FPLC - Ion exchange chromatography

The results from the chromatofocusing column implied that the GABP will bind to an anion exchange matrix even if the pH of the column buffer is lowered from pH 7 to pH 6 or 5.5. By lowering the pH, those proteins which have a pI near the pH of the column buffer would show little binding to the matrix. Such proteins could thus be easily excluded from the mixture to be purified.

The elution profiles of DE32 eluate on a Mono Q - FPLC ion exchange column at pH 7 and 5.9 are shown in Figure E9. The assay of the eluted fractions (Tables E11 and E12) confirms that no GABP elutes in the unbound fractions at either pH. However, specific binding was observed over a broad range of the bound fractions. GABP eluted with 0.18 to 0.43 M KCl at pH 7.0, and 0 to 0.37 M KCl at pH 5.9. Bound fractions did not show enhanced specific binding compared to the DE32 eluate. Similar results were obtained at pH 5.5 and 5.0, even when step gradients were used to ensure complete separation of protein fractions (data not shown).

Protein recovery from the Mono Q column was generally near 50%. Some of the losses may have been due to protein

Figure E9. Chromatography of DE32 eluate on Mono Q ion-exchange column at pH 7.0 (a) and pH 5.9 (b).

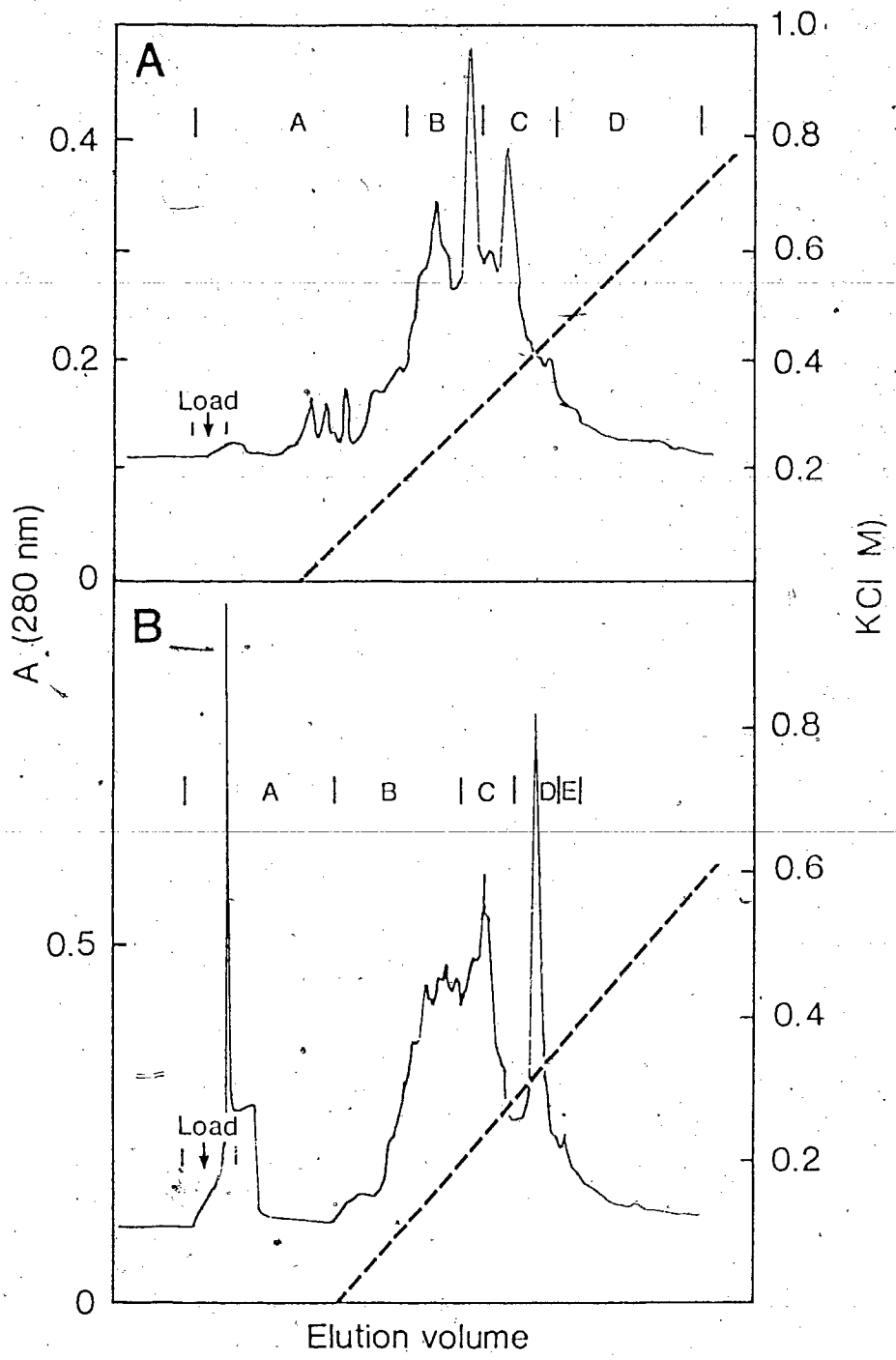

A
A
B
C
D
0.4
0.2
0
Load
1.0
0.8
0.6
0.4
0.2
B
A
B
C
D
E
0.8
0.6
0.5
0.4
0.2
0
Load
A (280 nm)
KCl M)
Elution volume

Table E11. [^{3}H]GA_4 binding to DE32 eluate and fractions eluted from ion exchange column (Mono Q) at pH 7.0.
Fractions were collected according to Fig. E9.a and assayed with Amersham [^{3}H]GA_4.
Data represent [^{3}H]GA_4 bound (pmol·mg^{-1} protein).

Incubation	DE32	Mono Q eluting KCl concentration (M)			
		0-0.18 A	0.18-0.30 B	0.30-0.43 C	0.43-0.67 D
10 nM [^{3}H]GA_4	0.18	0.04	0.20	0.22	0.02
+ 1 μM GA_4	0.10	0.02	0.13	0.15	0.01
+ 1 μM GA_3	0.11	0.02	0.14	0.15	0.01
+10 μM GA_4ME	0.15	0.02	0.19	0.18	0.01

Table E12. [^{3}H]GA_4 binding to DE32 eluate and fractions eluted from ion exchange column (Mono Q) at pH 5.9.
Fractions were collected according to Fig. E9.b and assayed with Amersham [^{3}H]GA_4.
Data represent [^{3}H]GA_4 bound (pmol·mg^{-1} protein).

Incubation	DE32	Mono Q eluting KCl concentration (M)				
		unbound A	0-0.20 B	0.20-0.30 C	0.30-0.37 D	0.37-0.40 E
10 nM [^{3}H]GA_4	0.18	0.13	0.15	0.19	0.20	0.08
+ 1 μM GA_4	0.12	0.09	0.10	0.12	0.13	0.06
+ 1 μM GA_3	0.14	0.08	0.11	0.15	0.14	0.05
+10 μM GA_4ME	0.15	0.09	0.13	0.17	0.15	0.06

precipitation on the column. This precipitation which was more apparent at acid pH values was further investigated.

e. pH fractionation

The precipitation of some proteins in the DE32 eluate at acid pH, suggested a possibility for differentially precipitating proteins at different pH values. For this purpose, DE32 eluate in buffer B was equilibrated with an acidic buffer using a small Sephadex G-25 column. The pH of the eluate was readjusted to the desired acidity, and precipitates formed were removed by centrifugation. The pH of the supernatant was lowered again and the process repeated. Proteins in the final supernatant were collected by $(NH_4)_2SO_4$ precipitation. All pellets were assayed at pH 7.0.

The results of the in vitro assay, performed by Ms. Joan Chisholm, indicated that the GABP precipitates between pH 7.0 and 5.7 (Table E13). This was also confirmed with DE32 eluate prepared from seeds germinated for 24 h (Table E14). About 40 to 60% of the total protein mass remains in the pH 5.5 supernatant. Lack of specific binding in pellets obtained at pH values less than pH 5.5 does not appear to be related to denaturation, as active GABP was recovered from the chromatofocusing column between pH 5 and 4.

Table E13. Effect of pH on precipitation of GABP in DE32 eluate from hypocotyls. DE32 eluate was equilibrated into 20 mM piperazine-HCl, 5 mM DTT, pH 6.0 using a desalting column. The eluate was readjusted to pH 6.2. Precipitates formed were collected by centrifugation. The pH of the supernatant was lowered to the value shown and recentrifuged. This process was repeated several times. Proteins in the final supernatant (F.S.) were collected by $(NH_4)_2SO_4$ precipitation. All pellets were resuspended in buffer B at pH 7.0 prior to assay. Data shown represent Amersham [^{3}H]GA_4 bound (pmol·mg^{-1} protein).

Incubation	DE32	pH					F.S.
		6.2	5.7	5.1	4.6[a]	4.3[a]	4.3
10 nM [^{3}H]GA_4	0.06	0.08	0.06	0.02	0.02	0.01	0.03
+ 1 μM GA_4	0.02	0.03	0.02	0.01	0.01	0.01	0.01
+ 1 μM GA_3	0.05	0.07	0.05	0.03	0.01	0.01	0.02
+10 μM GA_4ME	0.06	-	0.06	0.03	0.01	0.01	0.01

[a] assayed at a protein concentration of <0.2 mg·ml^{-1}

Table E14. Precipitation of GABP in DE32 eluate from seeds at different pH values. DE32 eluate prepared from seeds that had been germinated for 24 h was used. Precipitation and assay of proteins in this extract was performed with the same procedure as described in Table E13. Data shown represent Amersham [^{3}H]GA_4 bound (pmol·mg^{-1} protein).

Incubation	DE32	pH			F.S.
		6.0	5.5	5.0[a]	5.0
10 nM [^{3}H]GA_4	0.06	0.12	0.11	0.01	0.03
+ 1 μM GA_4	0.01	0.04	0.03	0.01	0.03
+ 1 μM GA_3	0.04	0.09	0.06	0.01	0.02
+10 μM GA_4ME	0.05	0.12	0.08	0.01	0.03

[a] assayed at a protein concentration of <0.2 mg·ml^{-1}

f. Open column gel permeation chromatography

Sephadex G-75 (2.5x27 cm) and Sephacryl S-200 (2.5x52.5 cm) were tested for a molecular size-based purification of the GABP. The binding data obtained with column fractions indicated a molecular weight range for the GABP between 50 and 150 kdalton, supporting the results of Keith et al. (1981). This method had poor resolution, low sample loading capacity and slow speed. The use of these column matrices, therefore, did not seem to be practical at this stage.

Further attempts at GABP purification are described in Appendix 2.

III. Discussion

Specific GA binding has been detected in all cucumber tissues examined. Thus it is present in ungerminated seeds as well as in GA-responsive and -unresponsive seedling tissues. However, on a g^{-1} fresh weight basis, GA-sensitive apical hypocotyl sections of 7 day old seedlings have the highest GABP concentration (Table E1, Figs. E1 and E2, and also Keith et al. 1982). These results suggest that a tissue's GA sensitivity may not only be determined by receptor numbers but also by other factors, such as receptor activation, gene availability etc. (see Michell and Houslay 1986).

Although a number of methods were examined for the purification of GABP from cytosol, only a small enhancement of specific binding was achieved. $(NH_4)_2SO_4$ precipitation followed by open column anion exchange chromatography allowed the bulk fractionation of cytosol and removal of proteins that carry a net neutral or postive charge at pH 7 from the GABP. These steps resulted in only about 3-fold purification of the GABP (Table E7). Some further purification of this protein using a hydroxylapatite or a blue dextran-agarose column was demonstrated (Tables E8 and E9). Specific binding was detected in fractions bound by FPLC chromatofocusing or ion exchange columns. GABP could also be recovered from precipitates obtained after lowering the pH of DE32 eluate from pH 7 to pH 6.2 or 5.7. However, low recoveries and/or low enhancement of specific

binding with these methods have limited the progress.

Binding site degradation may be one factor contributing to the low degree of purification achieved. The similar chromatographic properties of the numerous proteins in the cucumber extracts is another. Thus the GABP coelutes with the majority of the proteins from a chromatofocusing column (Fig. E8). HPLC - gel filtration chromatograms (Fig. E5) and SDS-PAGE of partially purified GABP (Fig. E7) also demonstrate the complexity of these extracts and point to the expected difficulties in achieving high yield and high resolution GABP purification.

PART F

Conclusions

The development of an *in vitro* assay for GA binding proteins has allowed considerable progress in the slowly advancing field of GA receptors. In the present investigation factors influencing the detection of GABPs were examined. The assay allowed some characterization of the active site of a candidate receptor in cucumber hypocotyl extracts and was used for the development of a protocol for the partial purification of this protein. Several conclusions can be drawn from this work: Many nonreceptor entities bind GAs. The reliable detection of GA receptor-like proteins requires discrimination against such components. In Part F I introduced, therefore, measures to increase the ratio of specific to nonspecific binding. It was also deemed necessary to assess binding specificity of candidate protein fractions using a number of ligands of differing *in vivo* bioassay activity.

The filter assay is a nonequilibrium method and may not favor optimum GA - GA receptor interaction. Hence it will result in an underestimation of the affinity and number of binding sites in a sample.

In spite of these limitations, the *in vitro* assay allows the characterization of the GA binding site of GABPs in the absence of the effects of metabolism, compartmentation, or permeability barriers which complicate such analysis with *in vivo* assays. In Part D it was demonstrated that soluble GABPs in cucumber extracts bind GAs saturably and exchangeably. The binding protein shows great structural specificity for γ-lactonic C-19

GAs with a 3 β-hydroxyl and a C-6 carboxyl group. Such GAs are bound with high affinity. Additional hydroxylations of the A, C, or D ring of the ent-gibberellane skeleton or methylation of the C-6 carboxyl result in reduction or loss of binding affinity. These in vitro specificity data are generally supported by published in vivo bioassay data on the expected conformation of the active site of the GA receptor in cucumber.

The GABP is present in ungerminated seeds as well as target and nontarget tissues of cucumber seedlings (Part E). However, on a g^{-1} fresh weight basis extractable GABP activity is highest in apical hypocotyls, the tissue which also shows the highest in vivo sensitivity to GA application.

Overall, the GABP studied conforms with the characteristics expected of GA receptors. A modest degree of purification of this protein was achieved using $(NH_4)_2SO_4$ precipitation and DEAE chromatography followed by chromatography on a hydroxylapatite or a dye-ligand affinity column (Part E). However, further work on this aspect is a prerequisite to allow elucidation of the true cellular function of this protein.

PART G

APPENDIX 1

I. Review of the Rapid Molecular Effects of GAs Using Substrate Mobilization in Cereal Aleurone as a Model System

Research on the molecular basis of GA action has mainly focused on substrate mobilization in cereal grains. This system is reviewed in this chapter with a view to the potential role of GA receptors. Reviews of these and other morphogenetic and/or biochemical aspects of GA action can be found in Jacobsen (1983), Jones (1973), Letham et al. (1978), and Zeroni and Hall (1980).

The most detailed understanding of GA action has been gained from the cereal aleurone system in which GA can alter the pattern of RNA and protein synthesis. In cereal grains, GA released by the embryo controls the de novo synthesis and secretion of several hydrolytic enzymes in cells of the aleurone layer. These hydrolases mobilize endosperm nutrient reserves required by the developing seedling.

The most dominant hydrolase synthesized is α-amylase which in barley comprises a family of 2 groups of high and low pI isozymes. In addition, GA_3 stimulates the synthesis of β-glucanase, acid phosphatase, RNase, DNase, and several proteases (Hammerton and Ho 1986, Mundy et al. 1985). GA inhibits the synthesis of another seed protein, α-amylase/subtilisin inhibitor (Mundy et al. 1986). These actions of GA are generally inhibited by ABA.

A 3 to 8 h lag period between the application of GA_3 to deembryonated half seeds and the onset of α-amylase synthesis is usually reported (eg., Higgins et al. 1976, Jacobsen 1983). However, Muthukrishnan et al. (1983a) observed lag times as short as 1 h. A continuous presence of GA_3 is required throughout this lag period.

Cell-free translation studies have established that the appearance of the GA-induced proteins is preceded by increased levels of their corresponding de novo synthesized mRNAs. Increased transcript stability may also contribute to this effect. This has been shown using RNA isolated from whole cells, as well as protoplasts and nuclei prepared from oat and barley aleurone (Deikman and Jones 1986, Jacobsen et al. 1985, Jacobsen and Beach 1985, Zwar and Hooley 1986). While the levels of certain mRNAs increase in response to GA treatment, rRNA and total RNA transcripts decrease in barley aleurone (Jacobsen and Beach 1985). Muthukrishnan et al. (1983a), using cloned α-amylase DNA probes, detected α-amylase mRNA within 1 h of GA application. Levels of these mRNAs increased with increasing GA concentration. However, rapid mRNA induction, equivalent to that reported by Theologis et al. (1985), who observed auxin specific RNAs within 15 minutes of auxin application to pea epicotyls, have not been reported.

Several details about the effect of GA on transcription are noteworthy: All studies indicate that a cytoplasmic factor (receptor?) is required for GA to have an effect on gene

expression. GA-specific RNA transcripts are always isolated after whole aleurone cells or their protoplasts have been incubated in GA_3. Incubation of isolated nuclei in GA_3 has not been reported to result in a specific response. A similar requirement by nuclei for a soluble (cytoplasmic?) factor was noted by Johri and Varner (1968) for pea shoots. Difficulties in preparing undamaged nuclei could, however, also result in this phenomenon. Further support for the presence of GA receptors in aleurone tissue comes from the work of Keith et al. (1980) who observed saturable and specific binding of [^{3}H]GA_1 by barley aleurone. This work is discussed in more detail in Part A, 'Evidence for the Existence of GA Receptors'.

Differential regulation of the 2 groups of α-amylase isozymes which are encoded by 2 different sets of structural genes located on different chromosomes has been observed by Chandler et al. (1987) and Ho et al. (1987). They observed that the mRNA for the group with a high isoelectric point (pI) is induced by GA_3 within 3 to 4 h and reaches a maximum at 12 to 16 h before declining to low levels at 24 to 48 h. In contrast, the mRNAs for the low pI isozymes are present at low concentrations even in the absence of GA_3 but increase several-fold with GA_3 treatment. High levels of these mRNAs persist for at least 48 h (Chandler et al. 1987). The GA-receptor may, therefore, interact with several chromosomal sites.

Work by Muthukrishnan et al. (1983b) suggests that induction of α-amylase mRNA by GA requires one or more concomitantly

synthesized protein(s), as inhibition of protein synthesis by cycloheximide results in a drastic decrease in α-amylase mRNA levels even when GA is present. No further reports on this potential regulator appear to have been published.

The role of ABA in these processes is not clear. ABA inhibits GA-promoted changes in gene transcription (Jacobsen and Beach 1985, Zwar and Hooley 1986). Recent work from the laboratories of Ho and Jacobsen suggests that ABA induces the synthesis of a protein that inhibits GA-induced mRNA synthesis (Chandler et al. 1987, Ho et al. 1987). Furthermore, ABA may also influence GA-induced protein synthesis by having minor effects on post-transcriptional processes (Higgins et al. 1982). ABA action on the GA receptor appears to be unlikely.

In addition to controlling gene transcription, GA may also influence the synthesis and/or turnover rates of intracellular membranes, especially the ER, and thereby affect protein synthesis and secretion. Evidence for such action is, however, mostly circumstantial. Thus Collins et al. (1972) reported increased ^{32}P incorporation into cytidine triphosphate within 30 min of GA application. The authors suggested, but did not demonstrate, an involvement of these nucleotides in membrane biosynthesis. Activation, independent of de novo protein synthesis, of phosphorylcholine-cytidyl transferase and phosphorylcholine-glyceride transferase has been observed in barley aleurone within 2 h after GA treatment (Johnson and Kende 1971). These enzymes catalyze the incorporation of

phosphatidylcholine (lecithin) into membranes. *In vitro* activation of these enzymes occurs within minutes after GA_3 application and is inhibited by ABA (Ben-Tal and Varner 1974). GA-promoted increases in lecithin incorporation into ER membranes have been reported by Evins and Varner (1971) and have been attributed to an increased turnover rate of membrane constituents (Johnson and Kende 1971). These events have been reported to be followed by increases in rough ER (rER) and polysomes starting 4 h after GA treatment (Jones 1969, Vigil and Ruddat 1973). The rER has been associated with the synthesis of secreted proteins such as α-amylase (Jones and Jacobsen 1982). Some of the nonsecreted α-amylase isozymes may be produced in the polysomes. However, a direct effect of GA on rER and polysome synthesis as suggested by Collins *et al.* (1972) has been questioned by the biochemical studies of Rodaway and Kende (1978). Also, Gibson and Paleg (1976) observed no GA-induced changes in the levels of cytochrome C reductase, a marker of ER membranes, and Zwar and Jacobsen (1972) detected no significant changes in uridine and adenosine incorporation into rRNA due to GA. Colborne *et al.* (1976) criticized the work of Jones (1969) and Vigil and Ruddat (1973) for a lack of control micrographs of aleurones incubated in the absence of GA_3. However, their own ultrastructural study was characterized by a paucity of figures showing the effect of GA treatment. Nevertheless, Colborne *et al.* (1976) did show that extensive ER development occurs independent of external GA_3 application during early germination.

It is not clear whether the observed changes in membrane components are a direct consequence of GA action or that they are independent events accompanying other metabolic change. Whether GA directly affects rER and polysomes and thereby regulates post-transcriptional processes also remains unresolved.

A direct involvement of membrane lipids in GA action has been suggested by Singh and Paleg (1985). These authors examined dwarf wheat which contains a lesion at the Rht 3 gene. The response of this mutant to GA_3 as measured in terms of hydrolase production by aleurone and leaf elongation, is retarded (Ho et al. 1981). Singh and Paleg (1985) observed that this mutant has low levels of phosphatidylinositol, -choline, and -ethanolamine in the aleurone tissue compared to the normal, GA-responsive, tall wheat. Low temperature incubation or a 4 h IAA exposure of the dwarf aleurone prior to GA treatment resulted in increased synthesis of these phospholipids and an enhanced response to GA_3 (Singh and Paleg 1986). The authors suggested that the correlation between membrane lipid composition and GA_3-induced α-amylase synthesis points to membranes as determinants of GA sensitivity and sites of GA receptors. However, the authors did not examine the possibility that differences in gene availability, transcription, translation or ER membrane proliferation exist between the dwarf and tall cultivars. Such differences could affect the ability of the dwarf to respond to GA applications. In this context, it may be worth noting that

changes in membrane lipid composition can have profound effects on intrinsic membrane proteins (Carruthers and Melchior 1986).

The literature on the aleurone system clearly establishes that GAs can influence gene expression resulting in a specific hormonal response. A soluble or extranuclear receptor appears to be involved in the mechanism of GA action. Such a receptor remains to be purified and characterized. It is likely that the GA receptor interacts with a number of targets, such as different regulators of gene expression. The role of GAs in controlling cellular membrane organization remains unclear.

PART H

APPENDIX 2

The buffers referred to in this chapter were described in Part E, Materials and Methods.

I. Examination of Other *In Vitro* Assays for GABP

In addition to the DEAE filter assay a number of other methods for separation of bound from free ligand were examined with the hope of developing an alternative, sensitive technique for detection of GABPs.

1. *Sephadex G-25 minicolumn assay*

a. Materials and Methods

The assay procedure followed that described by Tuszynski *et al.* (1980). Sephadex G-25 (Pharmacia) was swollen in Buffer B and packed under gentle suction into 1 ml pipette tips (#230-1196 Y3K, Evergreen Scientific, Los Angeles CA) which had a small glass fiber plug at the outlet. These tips were chosen as their length exceeded that of other designs and allowed complete separation of a 100 µl mixture of Blue Dextran (eluted) and phenol red (retained) under conditions used for the assay. The minicolumns were fitted onto 10 x 130 mm test tubes and excess buffer was removed from the columns by centrifugation at 200*g*, 2 min.

Aliquots of incubation mixture were assayed by loading 50 or 100 µl aliquots onto the minicolumns at 2 C. The columns were then centrifuged at 200*g*, 2 min. Samples of the eluate were then collected and radioactivity measured as described previously.

b. Results

The results from the G-25 minicolumn assay were inconclusive (Table H1). Receptor-type binding appears to be detectable for 100 µl samples of G-25 eluate. However, data variation was very high; some data for replicates differed by one order of magnitude. These results have, therefore, questionable validity. The method was labor intensive and had many sources of variability. It was therefore abandoned.

2. Nitrocellulose filter assay

a. Materials and Methods

This assay is based on the ability of nitrocellulose membranes to bind proteins and with them bound ligands. The procedure of Inoue et al. (1983), who used cellulose ester membranes for the assay of thyroid receptors was adopted. Membranes (type HAWP, 0.45 µ, 2.5 cm diameter, Millipore Corporation) were soaked and then rinsed with 0.5 ml Buffer C under suction on a filtration manifold. Fifty µl samples of incubation mixtures, prepared as described previously, were then applied. This was followed by a wash with Buffer C volumes as shown in 'Results'. Membrane-bound radioactivity was measured by scintillation counting as described previously.

Table H1. Assay of GA binding to G-25 eluate using the Sephadex G-25 minicolumn assay.
Data represent average cpm eluted from columns. G-25 eluate was assayed at a protein concentration of 3.0 $mg \cdot ml^{-1}$.

Incubation	Sample volume	Buffer B	G-25 eluate
50 nM [^{3}H]GA_4		50	70
+ 5 μM GA_4	50 μl	120	70
+ 5 μM GA_3		30	140
+ 50 μM GA_4ME		70	80
50 nM [^{3}H]GA_4		390	1,060
+ 5 μM GA_4	100 μl	540	500
+ 5 μM GA_3		650	720
+ 50 μM GA_4ME		810	880

b. Results

On the basis of the results shown in Figure H1, a 120 ml wash volume was chosen to remove unbound [^{3}H]GA_4 from nitrocellulose filters. In a further experiment with these membranes the effect of sample volume on [^{3}H]GA_4 binding in the absence and presence of an excess of GA_4 was measured. Column buffer and G-25 eluate were used in the incubations. The presence or absence of cucumber extract did not greatly affect binding data over the volumes assayed (Fig. H2). Thus although total binding in the presence of G-25 eluate was about twice as high as in its absence, specific binding values did not differ significantly from background binding. The results suggested that under the conditions used very little GABP is bound by the membranes. This assay was therefore not pursued any further.

3. Polyethyleneimine-treated glass fiber filter assay

a. Materials and Methods

Bruns et al. (1983) have shown that acidic proteins (pI less than 7) are strongly bound by the polycationic polymer polyethyleneimine (PEI). They have used PEI coated glass fiber filters successfully for the assay of a number of animal receptor proteins. Their procedure was followed for testing its use for GABP assays. Whatman GF/B glass fiber filters (2.5 cm diameter) were soaked in 0.3% (v/v) PEI in water, pH 10, for 2 h before use and were placed on a filtration manifold without

Figure H1. Effect of wash volume on [^{3}H]GA_4 binding to nitrocellulose filter paper.
Fifty µl aliquots of incubation mixture containing 64 nM [^{3}H]GA_4 in Buffer B were loaded onto nitrocellulose filters. After 1 min, suction was applied and filters washed with different volumes of Buffer C.
Total cpm applied per filter = 153,000 cpm.

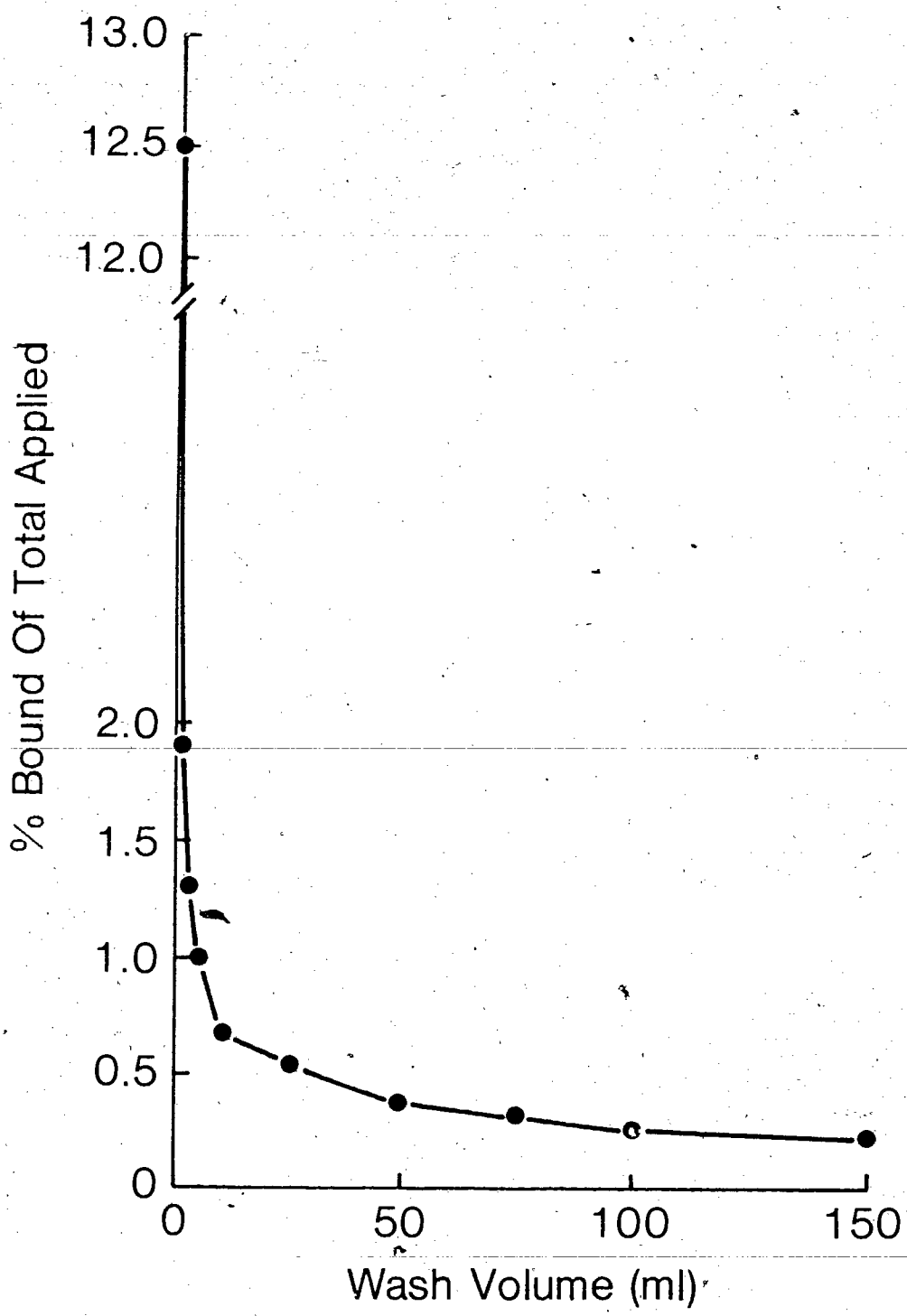
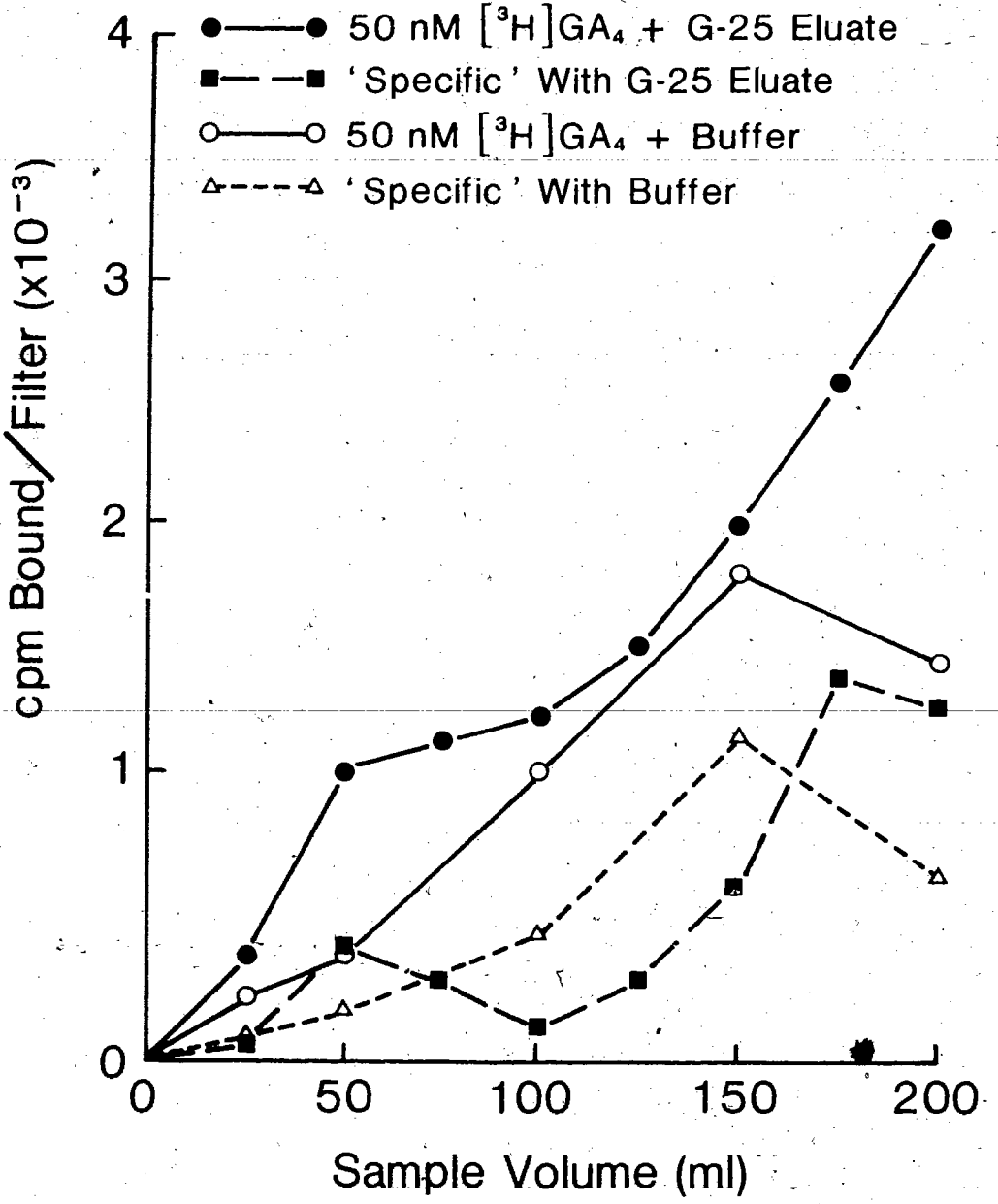

13.0
12.5
12.0
2.0
1.5
1.0
0.5
0
% Bound Of Total Applied
0
50
100
150
Wash Volume (ml)

Figure H2. Effect of sample volume and presence and absence of G-25 eluate on [^{3}H]GA_4 binding to nitrocellulose membranes. Samples of incubation mixtures containing 50 nM [^{3}H]GA_4 in the absence and presence of 5 μM GA_4 were applied to filter disks yielding total and nonspecific binding values, respectively. 'Specific' binding was determined by subtracting nonspecific from total binding.


50 nM [³H]GA₄ + G-25 Eluate
'Specific' With G-25 Eluate
50 nM [³H]GA₄ + Buffer
'Specific' With Buffer
cpm Bound/Filter (x10⁻³)
4
3
2
1
0
0
50
100
150
200
Sample Volume (ml)

washing. Duplicate 50 µl samples of incubations of 50 nM [^{3}H]GA_4 in buffer B or G-25 eluate, were loaded onto the filters. Various volumes of Buffer C were used as indicated in 'Results' to remove unbound radiolabel. Bound radioactivity was measured by scintillation counting as described previously.

b. Results

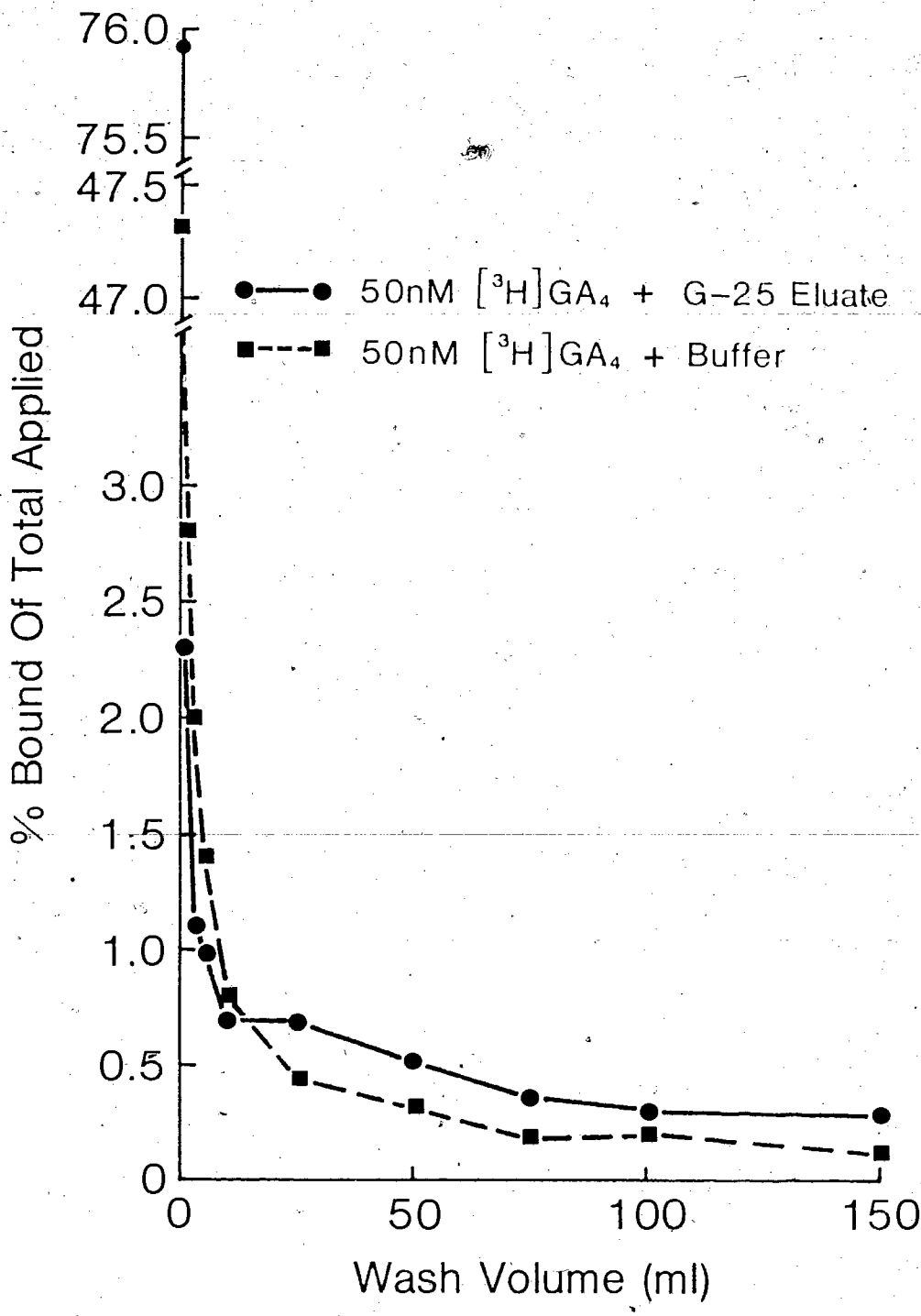
PEI-treated glass fiber filters also did not bind significant amounts of GABP (Fig. H3).

4. Ultrafiltration membrane assay

a. Materials and Methods

This method is based on an approach used by Venis (1985) for the assay of auxin binding proteins. Separation of bound from free hormone is achieved by passing the incubation mixture through a semipermeable membrane with defined pore size. Receptor-bound hormone is expected to be retained by the membrane while unbound GA is washed through. Discs (2.4 cm diameter) of the 2 types of ultrafiltration membranes, purchased from Amicon Canada Ltd., Oakville ON, were tested. PM20 (Lot 2960) and YM10 (Lot 2800) were used. According to the supplier the PM20 membrane is made from an inert, nonionic polymer which tends to bind hydrophobic molecules and has a molecular weight cut-off for globular proteins of 20 kdalton. Compared to the PM20 the YM10 membrane is more hydrophilic and has low protein binding properties. It has a molecular weight cut-off of 10

Figure H3. Effect of wash volume on [^{3}H]GA_4 binding to PEI-treated glass fiber filters in the absence and presence of G-25 eluate.
Fifty µl aliquots of incubation mixture were loaded onto filters and immediately washed with the volumes of Buffer C indicated.
Total cpm applied to each filter = 215,000 cpm.


76.0
75.5
47.5
47.0
3.0
2.5
2.0
1.5
1.0
0.5
0
% Bound Of Total Applied
50nM [3H]GA4 + G-25 Eluate
50nM [3H]GA4 + Buffer
0
50
100
150
Wash Volume (ml)

kdalton.

For the assay, 100 µl samples of incubation mixtures were loaded onto presoaked membrane discs under suction. The effect of different wash volumes as well as competitors was tested. Membrane-bound radioactivity was measured by scintillation counting as described previously.

b. Results

The PM20 ultrafiltration membranes seem to have a greater protein binding capacity and better flow properties than the YM10 filters. A wash volume of 2 ml appeared to be sufficient for the removal of unbound radiolabel (Fig. H4). This wash required about 1.5 min and was used for a follow-up experiment in which binding in the presence and absence of different competitors was assayed. Some GABP appears to be retained by the PM20 filter (Table H2). However, the difference between binding data in the presence and absence of G-25 eluate was quite small (compare with DEAE cellulose filters, Table C2). This ultrafiltration assay did, therefore, not appear very promising.

Figure H4. Effect of wash volume on [^{3}H]GA_4 binding to ultrafiltration membranes in the presence and absence of G-25 eluate.
Fifty nM [^{3}H]GA_4 was incubated in the presence and absence of G-25 eluate. Aliquots (100 µl) were loaded onto ultrafiltration membranes under suction and bound radioactivity determined.
Total cpm applied per filter = 250,000 cpm. With YM10 membranes, washing times for volumes greater than 0.5 ml exceeded 10 min and thus were not assayed.

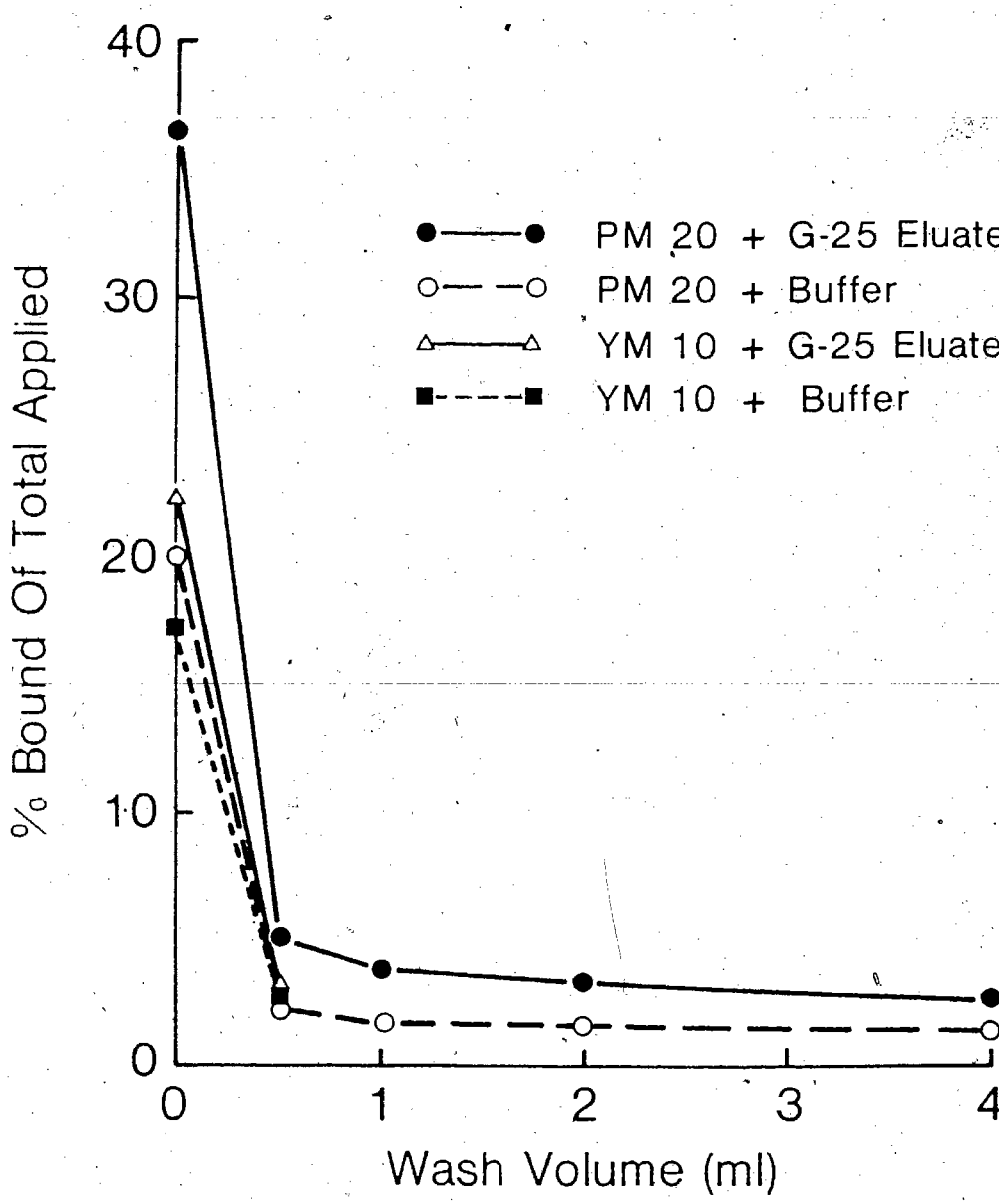

40
30
20
10
0
0
1
2
3
4
% Bound Of Total Applied
Wash Volume (ml)
PM 20 + G-25 Eluate
PM 20 + Buffer
YM 10 + G-25 Eluate
YM 10 + Buffer

Table H2. Competition for binding of [^{3}H]GA_4 to PM20 ultrafiltration membrane discs in absence and presence of G-25 eluate.
Soaked membrane discs were rinsed with 0.5 ml Buffer C. Aliquots of incubation mixture were passed through under suction and filters washed with 2 ml Buffer C to remove unbound radioactivity. Data represent cpm bound per 100 µl sample. G-25 eluate was assayed at a protein concentration of 3.6 $mg \cdot ml^{-1}$.

Incubation	Buffer B	G-25 eluate
50 nM [^{3}H]GA_4	500	690
+ 5 µM GA_4	350	490
+ 5 µM GA_3	390	480
+ 50 µM GA_4ME	320	570

II. Further Attempts at GABP Purification

1. Ultrafiltration

a. Materials and Methods

Two types of ultrafiltration devices purchased from Amicon Canada Ltd, Oakville, ON were tested for increasing the purity and/or concentration of the GABP. 'Centriflo' membrane cones, CF25 amd CF50A, (25 and 50 kdalton cut-off, respectively) were used in a centrifuge at 800g. Diaflo ultrafilters PM10 and XM100A (10 and 100 kdalton cut-off, respectively) were used in a model 8050 stirred ultrafiltration cell on ice.

b. Results

Receptor-type binding was retained by both 25 and 50 kdalton membranes, however, specific binding was not improved (Table H3). Displacement of [^{3}H]GA_4 by GA_4ME was increased in the cone fractions. This may reflect some changes in the configuration of the GA binding site of the GABP. No protein and no GABP-type binding was observed in the fractions not retained by the filters. Slow speed, limited capacity, and variability in flow rates between cones led to the discontinuation of their use.

10 kdalton stirred cell filters retained GABP. Total [^{3}H]GA_4 binding increased, data quality was however poor (Table H4). Results obtained with 100 kdalton stirred cell ultrafiltration membranes were also inconclusive.

Table H3. Binding of [^{3}H]GA_4 to DE32 eluate retained by 2 types of ultrafiltration cones.
Particulates were removed from DE32 eluate at 7,000g, 5 min. The supernatant was loaded into the cones with different molecular weight cutoff. The cones were repeatedly centrifuged at 800g and washed with buffer B.

Incubation	DE32 eluate	>25 Kdal	>50 Kdal
50 nM [^{3}H]GA_4	0.38	0.34	0.34
+ 5 μM GA_4	0.18	0.08	0.13
+ 5 μM GA_3	0.23	0.15	0.17
+50 μM GA_4ME	0.34	0.23	0.23

Table H4. Binding of [^{3}H]GA_4 to DE32 eluate retained by 10 Kdal ultrafiltration membranes.
Particulates were removed from DE32 eluate at 7000g, 5 min. The supernatant was loaded into a stirred ultrafiltration cell containing a 10 kdal ultrafiltration membrane. The sample was concentrated under pressure and repeatedly washed. Data represent pmol [^{3}H]GA_4 bound·mg^{-1} protein.

Incubation	DE32 eluate[a]	>10 Kdal[a]	>10 Kdal[b]
50 nM [^{3}H]GA_4	0.23	0.41	0.13
+ 5 μM GA_4	0.10	0.27	0.05
+ 5 μM GA_3	0.10	0.38	0.09
+50 μM GA_4ME	0.19	0.35	0.14

[a]assayed at a protein concentration of 1.2 mg·ml^{-1}
[b]assayed at a protein concentration of 3.7 mg·ml^{-1}

All types of ultrafiltration membranes had slow flow rates with the relatively crude protein solutions used. In the stirred cell system precipitates would form suggesting denaturation of proteins.

2. Nondissociating PAGE

a. Materials and Methods

Preparative, nondissociating PAGE was performed adopting the method of Abramovitz et al. (1984). A 1x12x0.3 cm stacking gel (3% T, 2.7% C in 0.038 mM Tris-HCl, pH 6.5) was cast on a pre-electrophoresed separating gel (12.3x12x0.3 cm, 6% T, 2.7% C) in Tris-glycine electrode buffer (0.192 M glycine, 0.05 M Tris, pH 8.6). This buffer was also used in the upper and lower buffer chambers and maintained at 1 C.

Lyophilized DE32 eluate was resuspended in buffer B, washed and reduced in volume on a C50A ultrafiltration cone. A portion of the >50 kdalton fraction was retained for assay, the rest (2.6 ml, 6 mg protein) was diluted with 0.6 ml sample treatment buffer and loaded onto the stacking gel.

A current of 230 mA (900 V) was applied for 15 min followed by 70 mA constant current for 30 min when the bromophenol blue (BPB) dye front had migrated 2.5 cm into the separating gel. The top 3.5 cm of the slab gel were divided into 5 strips which were inserted into 50 kdalton cut-off dialysis bags (Spectrapore 6, 3.4 cm, Spectrum Medical Industries, Inc., Los Angeles CA).

Also, a 1.5 cm wide strip in front of the BPB front was collected for 'Control'. Electroelution of proteins from the gel strips was performed with 150 V constant voltage at 2 C for 2 h. Gel fragments were removed by centrifugation. The supernatant was washed with buffer B and concentrated in a CF50A ultrafiltration cone for assay.

b. Results

The feasibility of recovering GABP from extracts fractionated on nondenaturing polyacrylamide gels was tested. DE32 eluate was loaded on preparative slab gels and electrophoresed 4 cm into the slab. Recovery of total proteins by electroelution followed by ultrafiltration was poor (25%). Table H5 shows that no GABP activity was recovered by this procedure. A follow-up experiment was performed. This indicated that this loss could have been due to GABP denaturation during exposure to the electrophoresis buffer. Table H6 shows that during the minimum time required to perform preparative electrophoresis, 5 h, considerable loss of specific binding occurs. The electrophoresis buffer used was at pH 8.6 but has an actual operating pH near 10 when used during electrophoresis. A number of other buffer systems which, according to Jovin et al. (1978) are supposed to operate near pH 7, were tested but none gave satisfactory protein mobility and banding patterns.

Table H5. Binding of [^{3}H]GA_4 to DE32 eluate before and after preparative PAGE.
DE32 eluate was assayed before and after preparative electrophoresis. A sample that was electroeluted from a 1.5 cm wide gel strip in front of the bromophenol blue dye front was used as a control. Data represent [^{3}H]GA_4 bound (pmol·mg^{-1} protein), except for control (pmol·ml^{-1}).

Incubation	control	DE32 eluate before PAGE	DE32 eluate after PAGE
50 nM [^{3}H]GA_4	0.33	0.51	0.47
+ 5 μM GA_4	0.26	0.25	0.40
+ 5 μM GA_3	0.25	0.33	0.41
+50 μM GA_4ME	0.27	0.44	0.40

Table H6. Effect of preparative electrophoresis buffer on [^{3}H]GA_4 binding to DE32 eluate.
DE32 eluate was assayed at time 0 (control) and after 5 h exposure to Buffer B or electrophoresis (PAGE) buffer. Data represent [^{3}H]GA_4 bound (pmol·mg^{-1} protein). Percentage [^{3}H]GA_4 displacement given in parenthesis.

Incubation	control	buffer B	PAGE buffer
50 nM [^{3}H]GA_4	0.40	0.51	0.35
+ 5 μM GA_4	0.16 (59)	0.20 (60)	0.26 (28)
+ 5 μM GA_3	0.29 (28)	0.25 (51)	0.28 (22)
+50 μM GA_4ME	0.34 (13)	0.35 (33)	0.32 (10)

PART I

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