Bot. Bull. Acad. Sin. (1995) **36:** 73–79

In vitro binding of gibberellin A_4 in epicotyls of dwarf pea

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(Received June 30, 1994; Accepted December 16, 1994)

Abstract. In vitro gibberellin (GA) binding properties of a cytosol fraction from epicotyls of dwarf pea (*Pisum sativum* L. cv. Progress No. 9) were investigated using [^{3}H]GA_4 in a DEAE filter paper assay at 4°C. The binding obtained is saturable, reversible, and temperature labile in dwarf pea, and has a half-life of dissociation of 5–6 min. By varying the concentration of [^{3}H]GA_4 in the incubation medium, the Kd was estimated to be 130 nM. The number of binding sites (n) was estimated to be 0.66 pmole mg^{-1} soluble protein. In competition binding assays, biologically active GAs, such as GA_3 and GA_4, could compete with [^{3}H]GA_4 for binding sites more successfully than could the biologically inactive GA_4 methyl ester or epi-GA_4. The influence of light on gibberellin-binding proteins in dwarf pea was also studied. The Kd and n of the GA-binding proteins for [^{3}H]GA_4 in light-treated tissues were close to the values for Kd and n in the dark grown tissues—light has little influence on gibberellin A_4-binding proteins.

Keywords: GA-binding proteins; Gibberellin; *Pisum sativum*.

Introduction

Gibberellin (GA) induced regulation of gene expression at both the mRNA and protein levels is well documented in the cereal aleurone system (Hammerton and Ho, 1986; Jacobsen and Beach, 1985), and several GA regulated genes have been isolated and characterized (Baulcombe et al., 1987). Synthesis of specific polypeptides in vivo and of translation products of extracted mRNA in vitro has been shown for GA-induced stem elongation in dwarf pea and corn (Chory et al., 1987). More recently, there is exciting progress in studies of auxin-binding proteins. Both the membrane bound and the soluble form of auxin-binding proteins have been identified and characterized (Bilang et al., 1993; Hicks et al., 1993), but there is little information on the early steps of GA recognition in the target region and on transduction of the hormonal signal into differential regulation of transcription.

It has been shown that [^{3}H]GA_4 binds to soluble protein fractions from cucumber and mung bean hypocotyls in vitro, and that this binding is specific for biologically active GAs but not for biologically inactive GAs, GA derivatives, or other phytohormones (Keith et al., 1981; Yalpani and Srivastava, 1985; Yalpani et al., 1989; Nakajima et al., 1993). Similar binding of [^{3}H]GA_1 to soluble fractions from dwarf pea epicotyls in vivo has also been shown (Keith and Srivastava, 1980), but aside from two preliminary communications, a specific binding in vitro has not been demonstrated nor have any kinetic data been provided. In this preliminary paper we report on specific binding of [^{3}H]GA_4 to soluble proteins from dwarf pea and provide kinetic data for such binding. We also investigate the influence of light on the gibberellin-binding proteins.

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Materials and Methods

Purification of Tritiated Gibberellin

The tritiated GAs were purchased from NEN (New England Nuclear, USA) as an unpurified mixture with a high concentration of side products. Our first purification step was preparative TLC on silica gel G plates (20 × 20 cm, 1.0 mm thick, Analtech), which separated the major side products from [^{3}H]GA_4 and its precursor, GA_7.

The partially purified tritiated GAs were further purified by reverse phase HPLC (Koshioka et al., 1983) on a preparative Partisil column (M9 10/50, ODS-2, 50 cm × 9.4 cm, Whatman) with different concentrations of methanol (Fisher, HPLC grade) for each run in 0.01 M phosphoric acid as the mobile phase. The radiopurity of the [^{3}H]GA_4 was checked by HPLC-RC with a Water Associate model 510 liquid chromatograph with a flow programmer equipped with a Lambda-Max 481 spectrophotometer and a radioactivity monitor (Ragtest, Ramona-LS). The purified tritiated GAs were also identified by GLC with a Hewlett Packard Gas Chromatograph 5790 A with a SE-30 column, a flame ionization detector, and a 3390 A integrator. Retention times were determined against a known [^{3}H]GA_4 standard (specific activity, 1.24×10^{12} Bq $mmol^{-1}$) purchased from Amersham, USA.

Extraction of GA-Binding Proteins

Pea seeds (*Pisum sativum* L. cv. Progress No. 9, Westcan Horticultural Specialist Ltd., Canada) were surface sterilized in 1% sodium hypochlorite and sown in vermiculite moistened with distilled water. Seedlings were grown in darkness for 8 days in a growth chamber at 25°C. The top 1 cm of GA responsive epicotyls were excised and pooled in ice-cold extraction buffer (pH 7.4) containing 100 mM phosphate buffer, 1.0 mM

ethylenediaminetetraacetic acid (EDTA), 50 μM phenylmethane-sulfonylfluoride (PMSF), and 5.0 mM dithiothreitol (DTT). The pooled epicotyls were weighed, and ground with an equal volume (1:1 w/v) of extraction buffer using a chilled mortar and pestle. All procedures were carried out at 4°C. The extract was filtered through four layers of cheese-cloth and centrifuged for 1.5 h at 100,000 *g*. The 100,000-*g* cytosol was cut with a saturated solution of ammonium sulfate to yield 60% ammonium sulfate. The pellets obtained were resuspended in a small volume of buffer (20 mM Tris-HCl, 1.0 mM EDTA, pH 7.0) and desalted on a Sephadex G-25 column (2.5 cm × 30 cm).

Reagents

Nonradioactive GA_4 (95% pure) was purchased from Abott Laboratory, USA. GA_3 (90% pure) was purchased from Sigma, USA. Epi-GA_4 and methyl ester of GA_4 (GA_4ME) were synthesized and purified by HPLC. All GAs were stored in an ethanol:ethyl acetate (1:1 v/v) mixture at -20°C.

DEAE-Cellulose Filter Assay

The DEAE-cellulose filter assay was performed as described in detail by Keith et al. (1982) and Yalpani and Srivastava (1985). [^{3}H]GA_4 in an ethanol:ethylacetate (1:1 v/v) mixture was put into 7-ml test tubes, along with other GAs or GA derivatives as required. The solutions were dried with N_2 gas, after which the tubes were cooled to 4°C. For the assay, 750 μl of 100,000-*g* cytosol or desalted protein fraction, each containing 1000–1500 μg of soluble protein, was mixed with dry [^{3}H]GA_4 to a final concentration ranging from 5 nM to 160 nM. After incubating on ice for 1 h, 100 μl samples were assayed for hormone bound to the protein using the DEAE-cellulose filter assay described below. For determination of tritiated GA that was nonspecifically bound, parallel incubations were carried out, in which a 100 or 1000-fold excess of nonradioactive GA or GA derivative was added as a competitor for the specific binding.

The filtration assay for bound [^{3}H]GA_4 was performed as follows: A stack of three DEAE-cellulose filter disks (diameter 2.2 cm, Whatman DE 81) was soaked in 10 mM phosphate washing buffer (pH 7.0) and inserted into a filtration manifold. The filter disks were rinsed with washing buffer, the vacuum was released, and a 100 μl sample was loaded. After exactly 1 min, 100 ml of washing buffer was drawn through the filter disks by suction to remove unbound [^{3}H]GA_4. The filter disks were allowed to run dry after the wash, and then were placed in scintillation vials containing 1 ml of ethanol. After 15 min, 8 ml of Scinti Verse II (Fisher, USA) was added. The vials were shaken and left to sit for a minimum of 1 h before measurement of radioactivity in a Beckman LS 8000 liquid scintillation counter. Counts were corrected for quenching, and the amount of specifically-bound [^{3}H]GA_4 was calculated by subtracting the activity bound in the presence of excess unlabelled hormone from that bound in its absence. The total [^{3}H]GA_4 concentration of the incubation was also checked by measuring the radioactivity of a 10 μl sample without filtration.

Results

Purification of Tritiated Gibberellin

The tritiated GAs from NEN were checked by GLC before purification by TLC and HPLC (Figure, 1A). The yield of tritiation was about 5% for [^{3}H]GA_4. After purification by TLC and HPLC, the purity of [^{3}H]GA_4 was more than 90% (Figure 1B). To make sure that this peak was [^{3}H]GA_4 and not some other radioactive compound, a run of [^{3}H]GA_4 spiked with standard [^{3}H]GA_4 purchased from Amersham was checked by HPLC-RC. It showed one peak with a retention time identical to [^{3}H]GA_4 (data not shown).

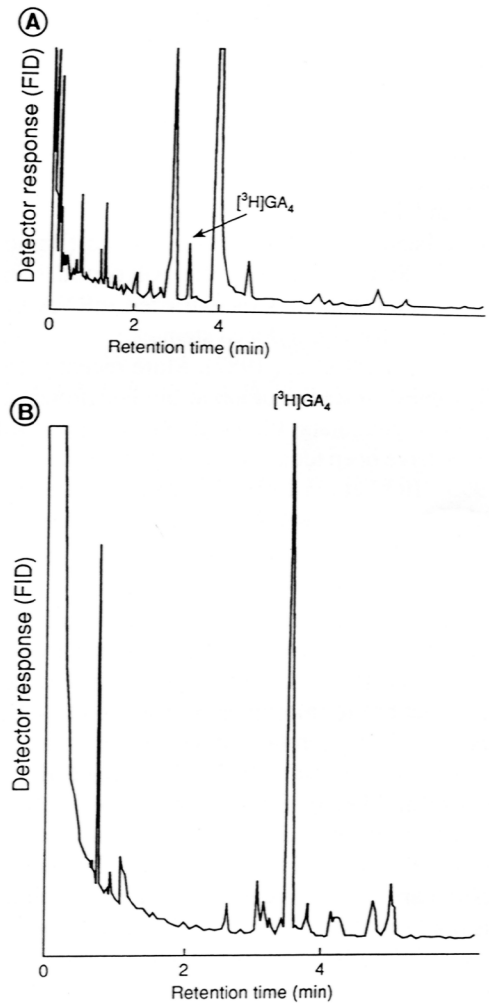

Figure 1. Gas-liquid chromatographs of the methylated and trimethylsilylated products after tritiation of GA_4. **A**, before purification; **B**, after purification by TLC and HPLC. Column, SE-30 (0.2 mm × 12 m); column temperature, 250°C; injector temperature, 260°C.

Rate and Reversibility of GA Specific Binding

The 100,000-*g* dwarf pea epicotyl cytosol was incubated for up to 24 h with [^{3}H]GA_4 with and without a 100-fold excess of nonradioactive GA_4. Samples were taken at various times and the total and specific binding was calculated. As shown in Figure 2, the specific binding reached an equilibrium after less than 2 h and remained stable for a minimum of 24 h. These results were similar to the data published for cucumber hypocotyl by Keith et al. (1981), except that those authors reported a slight increase in total binding and specific binding after 24 h incubation. To show that the [^{3}H]GA_4 binding was reversible, the 100,000-*g* dwarf pea cytosol was incubated with [^{3}H]GA_4, a 100-fold excess of nonradioactive GA_4 was added after 1 h, and the incubation continued for another hour. Samples were taken at various times and assayed for specifically-bound [^{3}H]GA_4. As shown in Figure 3, the half time of dissociation at 4°C was about 5 min and about 50% of bound [^{3}H]GA_4 was exchangeable with nonradioactive GA_4. We had previously established that the in vitro binding of GA_4 to extracts of dwarf pea epicotyl was disrupted by heat (data not shown), which suggested that the binding is to a protein fraction. Our current experiments established that [^{3}H]GA_4 binds in vitro at 4°C to a protein fraction and that it is exchangeable with nonradioactive GA_4. Specific binding of [^{3}H]GA_4 to partially purified protein, following ammonium sulfate precipitation and desalting of dwarf pea Cytosol, was also investigated at concentrations of [^{3}H]GA_4 ranging from 5 nM to 160 nM. Binding data were plotted according to the method of Scatchard (1949). As shown in Figure 4, the Kd was calculated to be 130 nM and n was estimated to be 0.66 pmole mg^{-1} soluble protein.

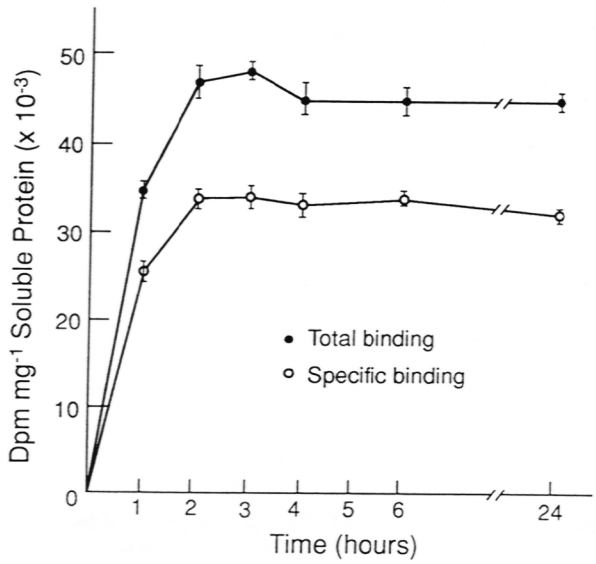

Figure 2. The influence of incubation period on levels of [^{3}H]GA_4 binding to ammonium sulfate precipitated and desalted protein from 100,000-*g* pea epicotyl cytosol that had been incubated in [^{3}H]GA_4 (50 nM) + 100-fold excess of nonradioactive GA_4. Error bars calculated from three measurements.

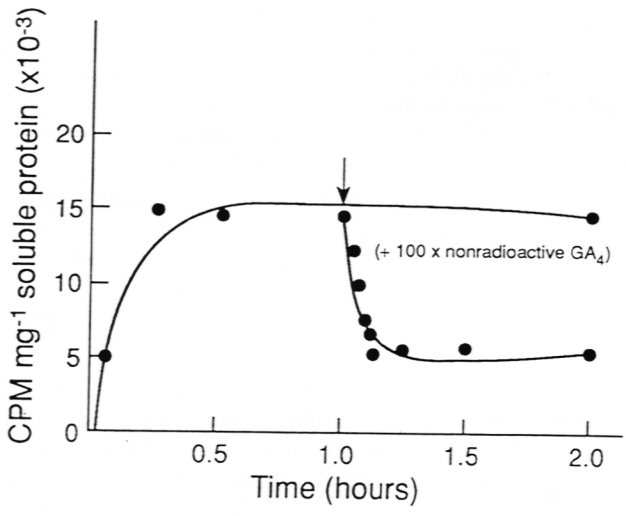

Figure 3. Total binding of 100,000-*g* pea epicotyl cytosol incubated in [^{3}H]GA_4 (100 nM) and the influence on the total binding of a 100-fold excess of nonradioactive GA_4 applied after 1 h (indicated by arrow).

Competition for the Specific Binding of [^{3}H]GA_4 by Other GAs

Early GA-binding papers reported that the biologically inactive GAs and GA methyl esters competed very little or not at all with specifically-bound radioactive GA (Keith

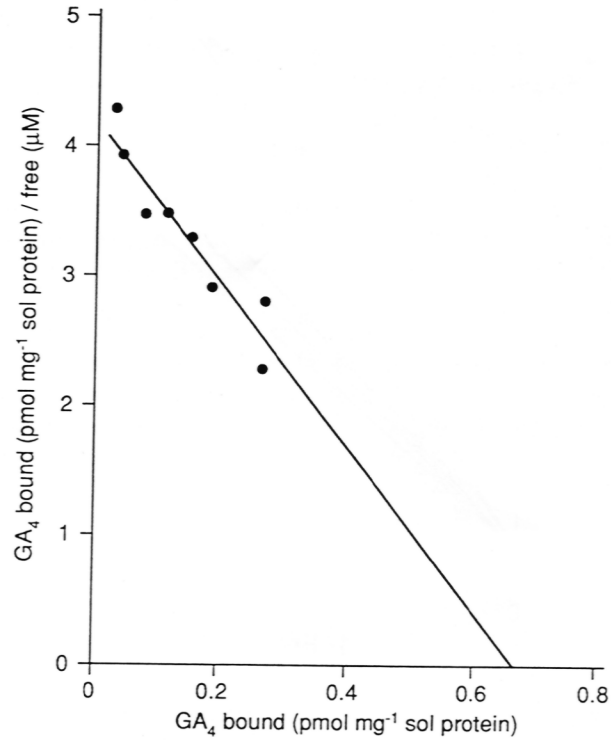

Figure 4. Scatchard plot of [^{3}H]GA_4 specifically bound to samples of ammonium sulfate precipitated and desalted protein from cytosol of pea epicotyl. Kd was estimated to be 130 nM, n was estimated to be 0.66 pmole mg^{-1} soluble protein.

et al., 1982; Yalpani and Srivastava, 1985). To confirm these results, specific binding of [^{3}H]GA_4 was studied in the presence of a 100 or 1000-fold excess concentration of nonradioactive GA_3, GA_4, epi-GA_4, and GA_4 methyl ester. The kinetics of binding were studied in the presence of fixed concentrations of competitors and varying concentrations of [^{3}H]GA_4, in competition experiments using dwarf pea cytosol. Double-reciprocal plots (Figure 5) indicated that these GAs competed for the same site. The biologically inactive epi-GA_4 and GA_4 methyl ester competed for the [^{3}H]GA_4 binding site, though much less than did biologically active GA_3 or GA_4. GA_1 is structurally similar to GA_3, but has a double bond between C_1 and C_2. Both GA_1 and GA_3 are biologically active, and hence the competition studies data can probably be extrapolated for GA_1 binding. The specific in vitro binding of [^{3}H]GA_4 in cytosol of dwarf pea epicotyl is in agreement with the published data from in vivo bioassays (Reeve and Crozier, 1974), and thus is biologically meaningful.

The Influence of Light on Gibberellin-Binding Proteins

Dwarf pea samples that had been transferred to light (Sylvania VHO cool white fluorescent tube, 150 μE m^{-1} sec^{-1}) for 24 and 120 h showed specific binding of 100,000-*g* cytosol to [^{3}H]GA_4. Figures 6 and 7 show the kinetics of [^{3}H]GA_4 binding. The Kd was estimated to be 100 nM for both 24 and 120 h light-treated pea cytosol. The number of binding sites (n) was estimated to be 0.22 and 0.18 pmole mg^{-1} soluble protein in the cytosol of 24 h and 120 h light-treated tissues, respectively, which is close to the Kd value for dark grown pea seedlings (130 nM). In vivo bioassays (data not shown) were also performed to ensure that there was a physiological growth response in pea seedlings to exogenous GA_4 after 24 and 120 h illumination.

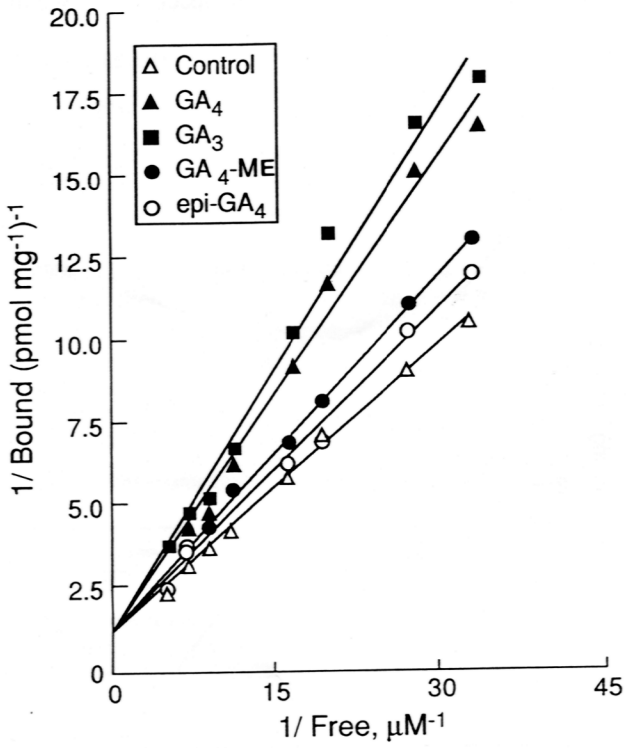

Figure 5. Double-reciprocal plot of [^{3}H]GA_4 binding to ammonium sulfate precipitated and desalted protein from pea cytosol in the presence of nonradioactive GAs. Samples were incubated with [^{3}H]GA_4 at a range of concentrations in the presence of GA_3, GA_4 (100 nM) and GA_4 methyl ester (GA_4-ME), and epi-GA_4 (0.4 mM). Data were evaluated by regression analysis.

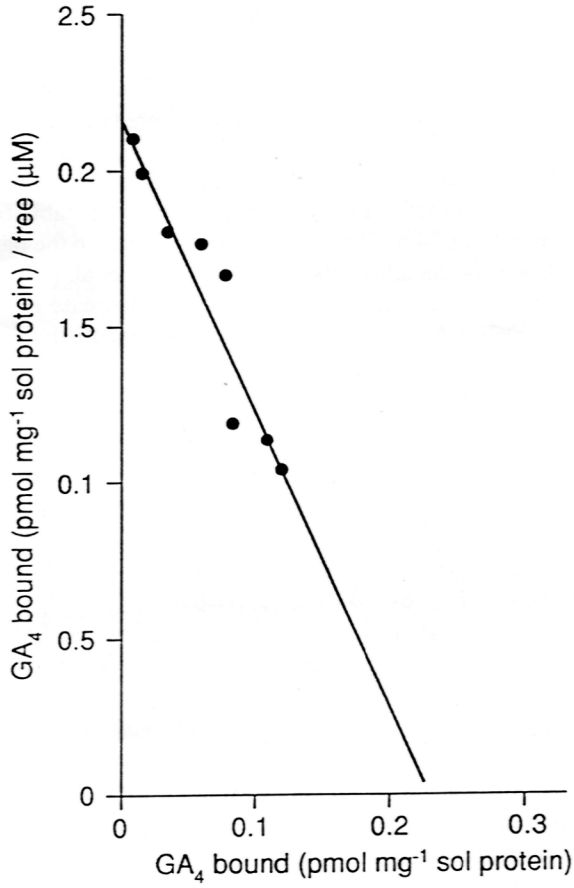

Figure 6. Scatchard plot of [^{3}H]GA_4 specifically bound to 100,000-*g* cytosol of dwarf pea seedlings that had been exposed to light for 24 h. Data were evaluated by regression analysis. Kd was estimated to be 100 nM, n was estimated to be 0.22 pmole mg^{-1} soluble protein.

Discussion

The specific binding of [^{3}H]GA_4 to protein fractions from dwarf pea is exchangeable, saturable, and stable for a minimum of 24 h. Keith and Srivastava (1980) and Keith et al. (1981) observed two classes of GA binding sites in pea and cucumber. The binding site with lower affinity (>1.0 μM) was detected at the high concentration of [^{3}H]GAs used for binding studies (400 nM to 2.0 μM). In the equilibrium studies, we used concentrations of [^{3}H]GA_4 ranging from 5 nM to 160 nM, and only one class of GA-

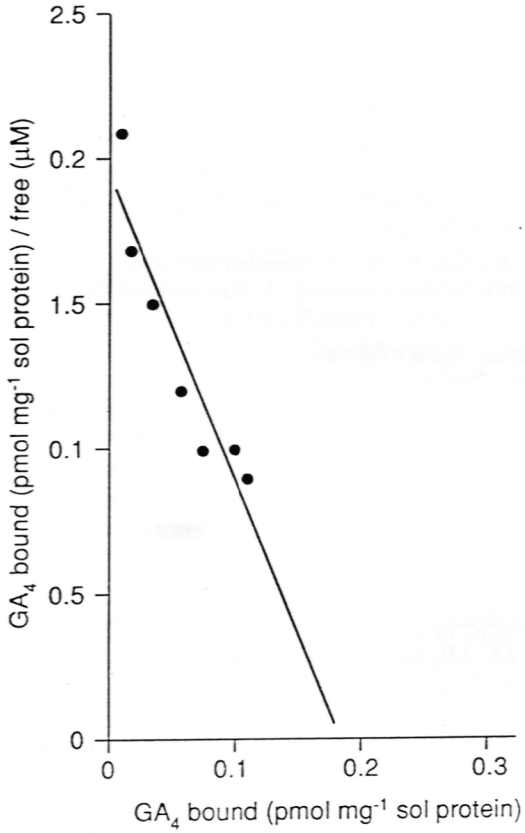

Figure 7. Scatchard plot of [^{3}H]GA_4 specifically bound to 100,000-*g* cytosol of dwarf pea seedlings that had been exposed to light for 120 h. Kd was estimated to be 100 nM, n was estimated to be 0.18 pmole mg^{-1} soluble protein.

binding protein with high affinity was observed in the cytosol and ammonium sulfate precipitated and desalted protein from pea epicotyl. The Kd was estimated to be 100–130 nM, which is lower than that of the GA_4 binding in mung bean hypocotyls (Kd, 300 nM) reported by Nakajima et al. (1993). Double-reciprocal plots of data from competition studies (Figure 5) show that at the high concentrations used, GA_4 methyl ester and epi-GA_4 also competed to some extent with [^{3}H]GA_4 binding at the same binding sites as GA_4. Our data are in partial agreement with the results of Yalpani and Srivastava (1985), who showed that the methylation of the C-6 carboxyl group of GA impedes or abolishes binding affinity. The decreased binding of GA methyl ester is related to its lack of biological activity (Crozier et al., 1970), which may be a result of GA_4 methyl ester being less polar than GA_4, or of the specific structural change in the methyl ester group at C_7. Serebryakov et al. (1984) reported that 7-homo-GA_3 (GA_3 with a -CH_2COOH group at C_6, and which has nearly the same polarity as GA_3) displayed only weak biological activity. This indicates that the negatively charged carboxyl group not only provides the ionized form, but also plays a part in the recognition of the hormone by the specific GA-binding proteins (possibly a positively charged amino group on the specific GA-binding protein molecule). The low specific binding of epi-GA_4 (Figure 5) and its total lack of biological activity are due to the conversion of the 3β-OH of the GA_4 molecule to the 3α-OH of the epi-GA_4 molecule. The data suggests strong hydrophobic interaction with the binding proteins in the vicinity of 3β-OH. The high ligand specificity and high affinity of GA-binding proteins observed in dwarf pea is good evidence supporting the belief that the binding is caused by the specific GA receptor proteins.

The Kd of the GA-binding proteins for [^{3}H]GA_4 in light-treated tissues (100 nM) is close to the Kd value in the dark grown tissues (130 nM). Similarly, the number of binding sites (n) in light grown tissue is close to the number of binding sites in the dark-grown tissue. These data indicate that light has very little influence on the affinity (Kd) for GA or on the number (n) of the GA-binding proteins. The hypothesis that light renders the plant tissues less responsive to given amounts of endogenous GAs by decreasing the amounts of GA-binding receptor molecules (here it is assumed that GA receptor molecules exist in the GA-binding proteins fraction) can be temporarily ruled out. Weller et al. (1994) suggested that light acts, in part, by constraining GA-signal transduction at a relatively late stage. The GA-binding properties examined in this preliminary study might be independent of the decreased responsiveness to light (Weller et al., 1994). An examination of the relationship between GA-signal transduction and GA-binding receptors affected by light is needed to elucidate the mechanism of light-induced growth inhibition in plants.

Using HPLC and GC-MS to separate and quantify the endogenous gibberellins in pea seedlings, Ingram et al. (1983) first showed that GA_1 was present in 20-day old light-grown plants of tall pea with the gene *Le*, and that it was absent, or present in undetectable levels, in dwarf genotype with the *le* allele. Ingram et al. (1984) also reported that labeled GA_{20} was metabolized to labeled GA_1 in tall pea, but not in dwarf pea. They concluded that the *Le* gene controlled the 3β-hydroxylation of GA_{20}. Similar results were reported in the mutants of maize by Spray et al. (1984). Campell and Bonner (1986) pointed out that the metabolism of GA_{20} was modulated by phytochrome, and suggested that the phytochrome Pfr form acted by preventing accumulation of effective levels of biologically active GA_1 through expression of the recessive *le* gene in light grown dwarf pea seedlings—this inhibits the activity of 3β-hydroxylase, and causes the dwarf growth habit (Ross et al., 1989). Therefore, the light-induced growth inhibition of pea stem may result from a decreased availability of biologically active GA_1 and the decreased responsiveness (Weller et al., 1994), which in turn is probably modulated by light. The decreased responsiveness of light-treated epicotyls to GA_1 may also result from the increased GA metabolic enzymes (Davies et al., 1986).

Acknowledgements. We thank professor W. E. Vidaver, L.M. Srivastava, and Dr. T.D. Ho, for their helpful discussions and suggestions for this paper.

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碗豆上胚軸之吉貝素 (GA) 受體結合蛋白

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利用陰離子交換樹脂之濾紙 (DEAE filter paper) 研究矮碗豆吉貝素 (GA) 結合蛋白，受體結合性質是可逆的、飽合的，並在高溫下呈現不穩定，分離半生期爲 5 - 6 分鐘，動力學之研究指出解離常數 (Kd) 約爲 130 nM ，結合數目估計爲 0.66 p mole 每毫克蛋白，競爭研究顯示具生物活性之 GA_3 和 GA_4 比不具活性之 epi-GA_4 及 GA_4-Methyl ester 對 GA_4 之結合蛋白質有較強之競爭能力，並且可能競爭同一結合位置，初步動力學研究亦顯示光照對 GA 結合蛋白之影響甚小。

關鍵詞：吉貝素(GA)；吉貝素結合蛋白；碗豆。