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Lalit M. Srivastava
Simon Fraser University

Konrad A. Sechley
Simon Fraser University

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In Search Of A Gibberellin Receptor

LALIT M. SRIVASTAVA and KONRAD A. SECHLEY[1]

Department Of Biological Sciences, Simon Fraser University, Burnaby, B.C. V5A 1S6

Use of tritiated gibberellins ([^{3}H] GA_1, [^{3}H] GA_4) of high specific activity and purity has allowed the determination of GA-specific binding sites in several plant tissues both *in vivo* and *in vitro*. In cucumber hypocotyls and pea epicotyls which have been most investigated the binding of [^{3}H] GA_4 occurs to soluble proteins. This binding is saturable ('n' = about 30 pmol. mg^{-1} soluble protein), of high affinity (K_D = about 70 nM), and is competed for by other GA_s and derivatives in proportion to their biological activity in these tissues *in vivo*. Size exclusion and ion-exchange chromatography has yielded several fractions which show specific and exchangeable binding of [^{3}H] GA_4, but further purification of these fractions and discrimination whether binding is to a GA metabolizing enzyme or the GA receptor has not yet been possible. Isolated nuclei from cucumber hypocotyls also show specific and exchangeable binding of [^{3}H] GA_4. They show run on transcription *in vitro*. Addition of 100 nM GA_4 to the transcription cocktail augments the total RNA produced and, as determined by sensitivity to α-amanitin, dramatically shifts the transcription in favor of RNA polymerase II activity. It also appears that the nuclei contain a soluble inhibitor of GA-induced transcription. GA-insensitive mutants, such as wheat varieties carrying the *Rht3* gene, provide a unique tool to study the mechanism of GA action. Preliminary data indicate, however that the D6899 wheat carrying the *Rht3* gene, is not a receptor mutant, rather it is a mutant which produces an inhibitor that prevents the GA-induced transcription of α-amylase genes in aleurone tissue. These and other data are reviewed with a backdrop of information about steroid receptors, and gibberellin biosynthesis and metabolism. A model of gibberellin action is presented which is consistent with published data, and some future lines of research are indicated.

INDEX DESCRIPTORS: [^{3}H] GA-binding, GA receptor, characteristics and purification; GA biosynthesis and metabolism; steroid receptors; nuclei, isolation, *in vitro* transcription; GA-insensitive mutants; mode of GA action.

Gibberellins are involved in regulating or controlling many different biochemical and morphogenetic responses (see Jones, 1973; Stoddart and Venis, 1980; Zeroni and Hall, 1983). The most investigated and best understood of these responses is that of the aleurone tissue of cereal grains where using intact tissue or isolated protoplasts it has been shown that exogenous application of GA_3 leads to marked changes in the fine structure and biochemical activities of the cell. Some 18 enzymes or enzyme systems are known to be affected; some are synthesized *de novo*, others show a stimulation in synthesis or activity, and still others show a decline in synthesis (see Jacobsen 1983). For some of these gene products correlated changes at the mRNA level have been documented (Chandler et al., 1984; Deikman and Jones, 1986; Zwar and Hooley, 1986; Nolan and Ho, 1988), which indicates that gibberellin action involves a differential regulation of expression of several different genes. Synthesis of GA-induced specific mRNAs and proteins has also been demonstrated during stem elongation in Dwarf pea, Dwarf corn (Chory *et al.*, 1987) and cucumber hypocotyls (Sechley and Srivastava, 1990), and gibberellin-induced inhibition of patatin accumulation has been shown in potato (Hannapel *et al.*, 1986).

Since at least some GA-mediated responses involve an up- or downward regulation of gene expression (see also Jacobsen and Chandler, 1987), the questions must be asked as to how the hormonal signal is perceived by the target cells and what are the events that lead to activation of some and inactivation of other genes. These early phases of gibberellin action are almost totally unknown. A model that is applicable to thyroid and many steroid hormones in animals and which on balance of available evidence we favour for gibberellins is that there are proteinaceous receptors in target cells which have a stereospecific recognition site for the hormone. On binding to the hormone, the receptor molecules are activated (or transformed) such that they can bind with higher affinity to specific nucleotide sequences, hormone response elements, HRE (or enhancers in the case of positive regulation), on the DNA to bring about an altered expression of the responsive genes. In the last few years there has been an explosion of information on steroid research and it is appropriate to review briefly the current status of steroid research as a back drop for a review on gibberellin receptors.

[1]Present Address: Department of Botany, University of Guelph, Guelph, Ontario N1G 2W1

Steroid and Thyroid Receptors

Much of the earlier work in the 60's and 70's on steroid and thyroid receptors centered on use of radioactive hormones to demonstrate specific, high affinity, and exchangeable binding of the ligand to a protein fraction or fractions. These studies were complicated and often gave ambiguous or contradictory results because the receptor proteins occur in small quantities and are highly labile and unstable in impure mixtures or under disruptive conditions. Nonetheless, these studies established the existence of the receptors, that they were proteins which were either loosely bound to nuclear matrix or occurred free in the cytoplasm, and that the hormone receptor complex interacted with the nuclear DNA (see Schrader *et al.*, 1981).

The purification of these proteins to homogeneity by conventional gel filtration and ion-exchange chromatography was not possible. Affinity chromatography using phosphocellulose and DNA cellulose columns improved purification (see Schrader *et al.*, 1977; Coty *et al.*, 1978; Scharder *et al.*, 1981), but significant advances in purification were made possible only by development and adoption of two other techniques: 1. Affinity chromatography using a natural or synthetic hormone (or analog) bound to a matrix with a suitable spacer arm. 2. Photoaffinity labelling where a suitably derivatized hormone or analog could be covalently linked to its receptor on irradiation with UV light; with such a linkage in place purification to homogeneity could be carried out under more rigorous and dissociative conditions without much risk of the loss of the marker ligand. (see e.g. Formstecher and Lustenberger, 1987; Gronemeyer and Govindan, 1986; Katzenellenbogen and Katzenellenbogen, 1988).

Using these purification techniques as well as analysis of amino acid sequences and proteolytic patterns, and molecular cloning, many steroid and thyroid receptors have been studied in detail and shown to have a basic similarity of design. Each has a domain specific to the hormone near the carboxyl terminal, a more conserved domain rich in basic amino acids and carrying the so-called zinc finger that binds to DNA, and a third more variable domain near the NH_2 terminal which is rich in acidic amino acids and believed to facilitate transcription (Gehring, 1987; Evans, 1988; Gronemeyer *et al.*, 1988; Green and Chambon, 1988). Availability of pure receptors and cloning of hormonally regulated genes have led to an elucidation of the precise molecular interactions between the hormone and the receptor, the activated receptor and the enhancer sequence or HRE as

well as transcription factors involved in gene regulation (Schüle *et al.*, 1988; Beato, 1989; Meyer *et al*, 1989; Miesfeld, 1989; Tsai *et al.*, 1988, 1989).

The location of the receptor protein in the target cell continues to be a matter of some speculation. An earlier model visualized the receptor protein to be localized in the cytoplasm. On binding to the hormone, the receptor protein was activated or transformed, moved to the nucleus, and brought about gene activation. An alternative model supported by radioautographic, immunocytochemical, and enucleation studies postulates that steroid receptors are predominantly, if not exclusively, intranuclear in location. Others have argued that cytoplasmic receptor sites cannot be excluded because the receptor protein must be synthesized. Also, there is considerable evidence for translocation of activated receptor from the cytoplasm to the nucleus (for further details, see Moudgil, 1987; Jensen, 1988; Barrack, 1988).

Biological Activity, Biosynthesis and Metabolism of Gibberellins

Several facts about the biological activity, biosynthesis and metabolism are relevant to a discussion of the mechanism of gibberellin action. Fortunately, these are some of the most investigated aspects of gibberellin research and several recent reviews are available (e.g., Crozier, 1981; Crozier and Durley, 1983; Graebe, 1987; Hedden, 1983; Hoad, 1983; Sponsel, 1987).

Some 72 naturally-occurring gibberellins are known, of which about 61 occur in vascular plants, about 25 in fungi, and 14 are common to both (see Sponsel, 1987). The structures of these GAs are known and their activities in different bioassays have been used to deduce those structural features of the GA molecule which are important for biological activity. Thus, the C-19, γ-lactonic GAs are active in higher plants and among them the 3-hydroxy (GA_4, GA_7) and 3,13-dihydroxy (GA_1, GA_3) GAs appear to be the most active (Fig. 1). These two groups of GAs have different orders of activity in different groups of plants. Thus, GA_4 and GA_7 (and the derivative 2,2-dimethyl GA_4) are much more active in hypocotyl elongation in cucurbits than in cereal aleurone system or elongation growth of epicotyls or hypocotyls in the pea family, while the reverse is true for GA_1 and GA_3 (Bearder, 1980; Crozier, 1981; Crozier and Durley, 1983). Several other GAs are reported to have a high or moderate activity in different bioassays (e.g., GA_5, GA_9, GA_{12}, GA_{36}, etc., see Crozier, 1981; but see the effects of metabolism below). Certain others, notably the 2β-hydroxylated GAs, such as GA_8, GA_{34}, GA_{51} are inactive in all bioassays. Conjugation of sugar residues at C-3, C-13, or C-7 renders GAs inactive. GAs are often stored in maturing seeds in inactive forms (see Sponsel, 1987). They also appear to be converted to inactive forms such as GA_8 when supplied in excess *in vivo* (see Musgrave *et al.*, 1972; Stoddart *et al.*, 1974; Keith *et al.*, 1980). Methylated derivatives, such as GA_1 methylester (methylated at C-7) are also inactive in most bioassays.

In bioassays, the biological response occurs over several decades of GA concentration (Kende and Gardner, 1976; Trewavas, 1982). This fact together with the observation that GA uptake by plant tissues *in vivo* is essentially unsaturable at room temperature (Musgrave *et al.*, *1972; Silk et al.*, 1977; see also Srivastava, 1987) has cast doubt on the existence of GA receptors (see Kende and Gardner, 1976;

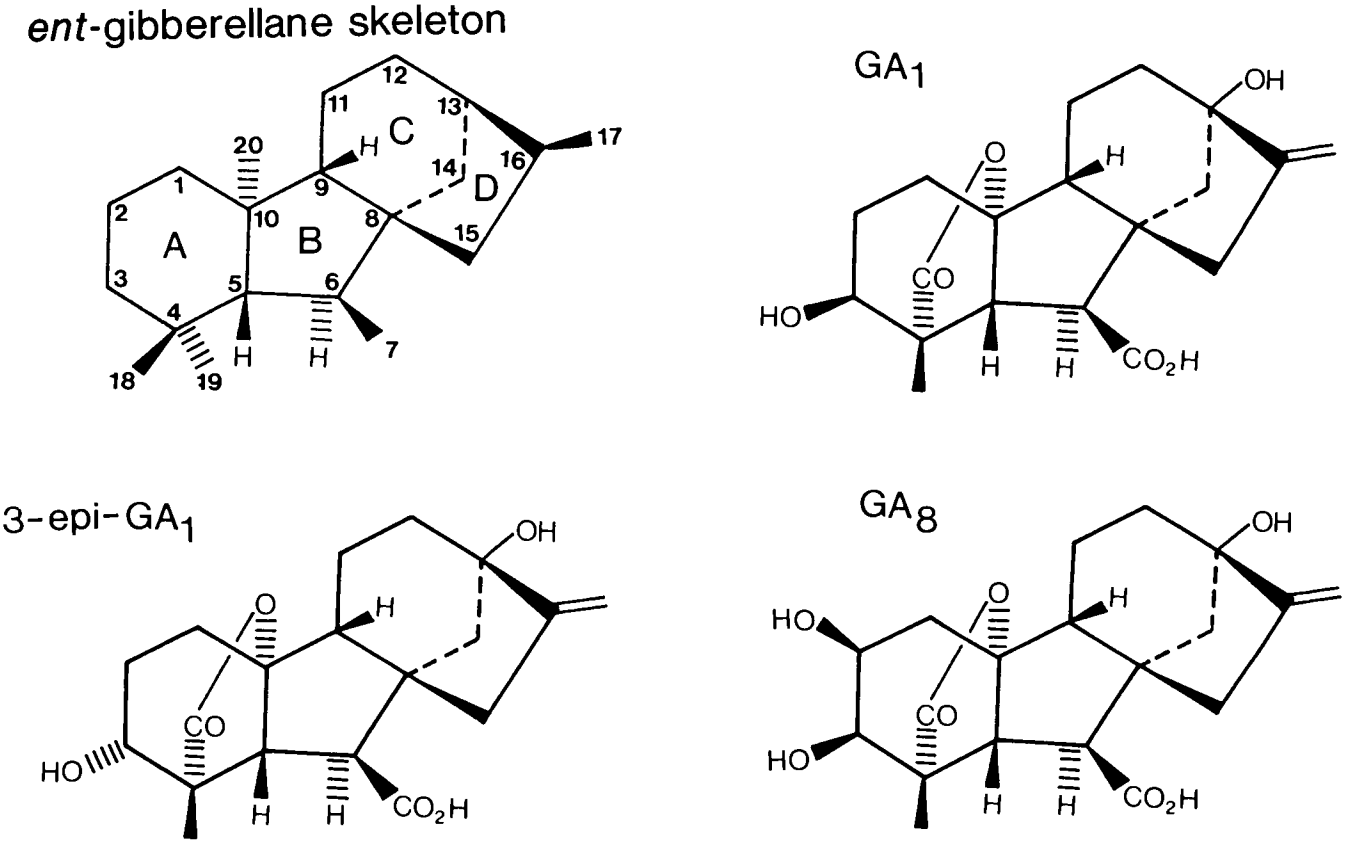

Fig. 1. Ent-gibberellane skeleton and structure of GA_1, 3-epi-GA_1 and GA_8. GA_1 is highly active in legumes and cereals, but 3-epi-GA_1 with a 3α-OH or GA_8 with a 2β-OH are both biologically inactive. GA_4 is similar to GA_1 but has no OH group at C13. It is highly active in cucurbits. (Adapted from Srivastava, 1987).

Stoddart, 1983). However, the dose-response curves and structure-activity data based on bioassays must be interpreted with a great deal of caution. The internal level of GA may bear little relationship to the concentration of GA supplied because of transport across living tissues; also, GA metabolism *in vivo* is known to lead to conversion of an inactive to an active GA or *vice versa* (e.g., Musgrave *et al.*, 1972; Stoddart *et al.*, 1974; Nash *et al.*, 1978; Gilmour *et al.*, 1984).

The biosynthetic pathways from mevalonic acid to GA_{12} aldehyde and subsequently to GA_4 or GA_1 are well established. In cell-free systems using radiolabeled precursors, some of the enzymes have been shown to be membrane bound (microsomal fraction) oxygenases requiring O_2 and NADPH whereas others occur in the soluble fraction and act as dioxygenases requiring O_2, Fe^{2+}, and 2-oxoglutarate in addition to NADPH for their activity (Takahashi *et al.*, 1986; Gilmour *et al.*, 1987; Graebe, 1987; Sponsel, 1987). The enzyme 2β-hydroxylase which converts GA_1 to GA_8 and 3β-hydroxylase converting GA_{20} to GA_1 have been partly purified and characterized (Smith and MacMillan, 1984, 1986; Kwak *et al.*, 1988). Both are 2-oxoglutarate dependent dioxygenases whose activity *in vitro* is stimulated by ascorbate. There is a suggestion that the activity of GA_{20} 3β-hydroxylase in the Dwarf pea mutant (*le le*) occurs at a higher rate in the dark than in the light and further that this regulation may be phytochrome mediated (Campbell and Bonner, 1988).

The existence of these various enzymes involved in GA synthetic or metabolic pathways must be taken into account in binding of radiolabeled GAs to a putative receptor (or receptors) and meaningful criteria must be established to distinguish binding of a labeled GA to a receptor protein on the one hand and enzymes on the other.

Criteria to Distinguish [^{3}H]GA Binding to a Receptor vs. Binding to an Enzyme

1. Among competing proteins, it may be assumed *a priori* that the receptor protein has an equal or higher affinity for the active GA than the biosynthetic or metabolizing enzymes. For GAs there is as yet no proof for this assumption and, indeed, the assumption may be incorrect. The binding protein in cucumber has a K_D of 70 nM for [^{3}H]GA_4 (Keith et al., 1982) which is very similar to the reported K_m for 2β-hydroxylase for [^{3}H]GA_1 in pea (Smith and MacMillan, 1986). The K_m values of 3β-hydroxylase from *Phaseolus* seeds for GA_{20} and GA_9, however, were 290 and 330 nM, respectively (Kwak *et al.*, 1988).
2. In a purification scheme the fraction containing the enzyme protein should show conversion of a substrate GA to its product under optimal conditions of temperature, pH, and cofactor requirements. Under similar conditions the fraction containing the receptor protein should show little or no conversion.
3. If a protein fraction shows binding to an active GA but no binding to its immediate precursor or derivative, the assumption may be made that binding is occurring to a receptor protein.
4. A proof that one is dealing with the receptor protein may be obtained by using a receptor mutant, supplying it with the receptor protein candidate and exogenous GA, and getting an unambiguous GA-induced mRNA or protein.

No putative plant receptor protein or protein fraction has as yet been shown to satisfy all the above criteria.

In vivo and *in vitro* gibberellin binding: —

In much of the earlier work on gibberellin receptors, saturability of [^{3}H]GA_1 uptake by intact plant tissues and binding of [^{3}H]GA_1 to macromolecular fractions could not be demonstrated (see Kende and Gardner, 1976; Srivastava, 1987). (Saturability of GA uptake is to be distinguished from "saturability" of GA-induced response.) Stoddart et al., (1974) showed specific binding of [^{3}H]GA_1 to a high molecular weight and an intermediate molecular weight fraction from Dwarf pea, but as in earlier studies (Musgrave et al., 1972), a large amount of [^{3}H]GA_1 taken up was converted to the inactive GA_8. The breakthrough came with the realization that saturability of [^{3}H]GA uptake by plant tissues *in vivo* could only be obtained if uptake experiments were done at temperatures which stopped or minimized GA metabolism. Uptake of [^{3}H]GA_1 by barley aleurone layers at different temperatures showed that saturation was obtained at 1.0 and 1.5°C but not at 3 and 4°C (Fig. 2). An analysis of metabolites showed that aleurone layers incubated at 1.0 and 1.5°C had only [^{3}H]GA_1 whereas at 3 and 4°C there was substantial metabolism of tritiated GA_1 to GA_8 and other polar metabolites. These experiments also showed that at equilibrium there was a higher concentration of [^{3}H]GA_1 inside the cell than in the ambient medium which suggested binding to subcellular components. Additional experiments revealed that the binding could be competed for by biologically active GA_1 but not by the inactive GA_8 (Keith et al., 1980). Experiments using cut slices of Dwarf pea epicotyl and cucumber hypocotyls gave similar evidence for saturable and exchangeable binding of [^{3}H]GA_1 and [^{3}H]GA_4, respectively, *in vivo*, and it was further shown that the tritiated GAs were being bound to a soluble protein or protein fractions (Keith and Srivastava, 1980; Keith *et al.*, 1981).

Subsequently, methodologies were developed to show *in vitro* binding of tritiated GAs to soluble protein fractions from a variety of plants. The methodologies for sample preparation and the binding assays used are given in Appendix 1.

The most detailed investigations to date have been on cucumber hypocotyl using the DEAE-filter paper assay. It has been shown that the binding of [^{3}H]GA_4 to 100,000 xg cytosol (or protein fractions therefrom) was saturable and exchangeable with nonradioactive GA_4 (half-life of dissociation, 6-7 min at 0-2°C). Scatchard plots using [^{3}H]GA_4 concentrations from 6 to 600 nM revealed a single class of binding sites with K_D of about 70 nM and number of binding sites (n) to be about 0.4 pmol.mg^{-1} soluble protein (Keith et al., 1982). There was a good correlation between the binding affinity of the protein for different GAs and GA derivatives and their biological activity in the cucumber hypocotyl bioassay (Yalpani and Srivastava, 1985). Thus, the binding protein had the highest affinity for GA_4, GA_7, and 2,2-dimethyl GA_4; GA_1 and GA_3 showed about 50 to 100 fold lesser affinity (the reverse is true for the binding protein in Dwarf pea), and GA_8, GA_{26}, 3-epi-GA_4, and GA_4 methyl ester showed little or no binding affinity. There were some notable exceptions. The binding protein showed little affinity for GA_9 and GA_{36}, both highly active in cucumber bioassay. This was all the more significant becase both are believed to be in the biosynthetic pathway of GA_4, and GA_9 may be its immediate precursor (see Hedden, 1983; Kwak *et al.*, 1988). It appears therefore that GA_9 and GA_{36} are metabolized to the active form, GA_4, *in vivo*.

Liu and Srivastava (1987) used the same assay to investigate [^{3}H]GA_4 binding to 100,000 xg cytosol from Dwarf and Tall pea. It was shown that [^{3}H]GA_4 binding was saturable, exchangeable with nonradioactive GA_4 and disrupted by heat. The K_D for [^{3}H]GA_4 was estimated to be 130 nM in Dwarf pea and 70 nM in Tall pea. The number of binding sites was estimated to be 0.66 and 0.43 pmol.mg^{-1} soluble protein in Dwarf pea and Tall pea, respectively.

The DEAE-filter paper assay gives an overestimate of K_D and an underestimate of n because the measurements of bound radioactivity are made under nonequilibrium conditions induced by filtration and washing. The calculated values of K_D vary between different experiments but are within the ranges expected from GA concentrations required for maximal biological response and represent high affinity binding. The values for n vary considerably and depend on degree of purification. For the 100,000 xg cytosol, the n correlates well with estimates of endogenous GA concentrations (see Srivastava, 1987).

Unfortunately, the DEAE filter paper assay is unsuitable for use

with [^{3}H]GA_1 (see Appendix 1). Lashbrook *et al.* (1987) used Sephadex chromatography to investigate binding of [^{3}H]GA_1 to 100,000 xg cytosol and protein fractions from dark grown Dwarf pea epicotyls. Whereas very little specific binding was observed to the crude 100,000 xg cytosol, the concentrated protein fraction from the intermediate molecular weight range (about 56 Kdalton MW) showed specific binding of [^{3}H]GA_1 which was saturable, pH sensitive, and could be exchanged with biologically active GA_1 and GA_4 but not by GA_{13}, GA_{17} or ABA.

Keith and Rappaport (1987) studied [^{3}H]GA_1 binding in the normal corn (Golden Jubilee) and the GA-sensitive (d_1, d_2, d_3, d_5) and GA-insensitive (D_8) dwarf mutants. The data for normal corn were not conclusive though there was some evidence for specific binding and a suggestion that the binding protein had aggregated to a larger molecular weight complex after binding to [^{3}H]GA_1. In steroid research, binding to the hormone is known to disaggregate the cytosolic protein to smaller units (see Jensen, 1988). The 100,000 xg cytosol from all dwarf varieties gave identical elution profiles after ion-exchange chromatography and using the d_1 corn it could be shown that fractions eluting at about 20, 30, 50 mM NaCl showed significant bound radioactivity.

In several of these studies it has been shown that the tritiated GA bound is the authentic [^{3}H]GA_1 or [^{3}H]GA_4 originally supplied and not a metabolite (Keith *et al.*, 1981; Keith and Rappaport, 1987; Liu, (1988). Liu (1988) used [^{3}H]GA_1 and cytosolic extracts from Dwarf pea seeds together with necessary cofactors, appropriate pH and temperature to demonstrate 2β-hydroxylase activity but even after incubations of up to 4 h at 25°C no conversion of [^{3}H]GA_1 to [^{3}H]GA_8 was seen (see also Keith and Rappaport, 1987). These negative results do not mean the absence of 2β-hydroxylase, merely that under the experimental conditions used [^{3}H]GA_1 was not converted to [^{3}H]GA_8.

The studies reviewed above indicate that under appropriate conditions of extraction and assay, specific, exchangeable and high affinity binding of [^{3}H]GA_1 or [^{3}H]GA_4 to cytosolic extracts can be demonstrated. This binding occurs to a soluble protein, is disrupted by heat, and is pH sensitive. Partly purified and concentrated protein fractions from the 100,000 xg cytosol give much better evidence of [^{3}H]GA binding than dilute, impure extracts. However, kinetic data on number of binding sites and binding affinity are available todate only for a limited number of cases. The data obtained so far do not exclude binding to enzymes; such binding probably does occur in some fractions. However, several facts support the conclusion that binding is occurring to a receptor protein: 1. precursors of GA_4, such as GA_9, GA_{36}, show no competition; 2. 2,2-dimethyl GA_4 competes strongly but 2β-hydroxylated GA_8 does not compete; 3. concentra-

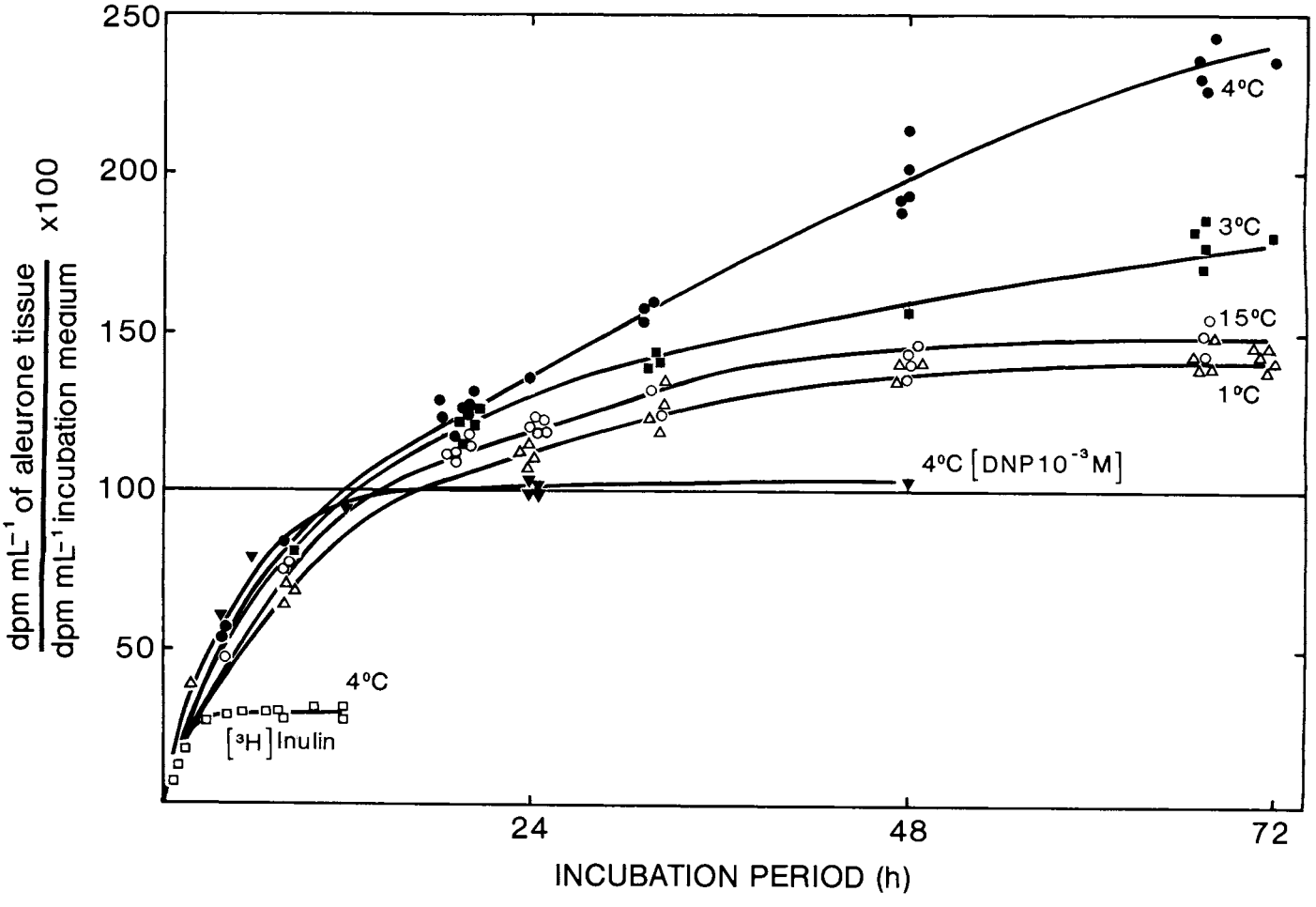

Fig. 2. The effect of temperature on the uptake of [^{3}H]GA_1 into barley aleurone layers. Concentration of [^{3}H]$GA_1 = 4.4 \times 10^{-9}$ M (41.5 Ci mmol^{-1}). Uptake of [^{3}H]inulin into free space, and uptake of [^{3}H]GA_1 (4.4×10^{-9}M) in the presence of DNP (1 mM), both at 4°C, are also shown. Level of [^{3}H]GA_1 activity in incubation medium = 100% = 415×10^3 dpm mL^{-1}. Level of [^{3}H]inulin activity in incubation medium = 100% = 60×10^3 dpm mL^{-1} (adapted from Keith et al., 1980).

Table 1. [^{3}H] GA_4 Binding In Dwarf Pea[a]

MATERIAL	K_D (nM)	n (pmol.mg^{-1} protein)
Apical part of epicotyl (target region)	140	0.28
Basal part of epicotyl (nontarget region)	120	0.21
Seed (24 h soaked)	160	0.14
Seedling (4 d old)[b]	170	0.28
Seedling (8 d old)[b]	165	0.29
24 h light-treated[c]	100	0.18
120 h light-treated[c]	100	0.22

[a]Data from Liu (1988).

[b]Dark grown for 4 or 8 days.

[c]Dark grown for 8 days, then exposed to light for 24 or 120 h.

tions of GA_1 or GA_3 required to displace [^{3}H]GA_4 binding in cucumber or Dwarf pea are consistent with binding to a receptor protein than to an enzyme.

Gibberellin Receptors in Nontarget Regions and at Different Stages of Plant Development:—

There is very little information on receptor-type binding in nontarget regions and at different stages of plant development. Both in cucumber and in Dwarf pea specific [^{3}H]GA binding has been reported in the non GA-responsive basal parts of hypocotyl or epicotyl (Stoddart *et al.*, 1974; Keith and Srivastava, 1980; Keith *et al.*, 1981, 1982). Liu (1988) found no significant differences in the number of binding sites or K_D for [^{3}H]GA_4 in the apical or basal parts of Dwarf pea epicotyls (Table 1). He also studied changes in binding kinetics for [^{3}H] GA_4 during seed germination of Dwarf pea and on transfer of dark grown seedlings to light. Whereas the K_D of the binding protein remained the same, there was a doubling in the number of binding sites between 24 h imbibed seeds and 4 day old dark grown seedlings (Table 1). Transfer of 8 d old dark grown seedlings for 24 or 120 h in light did not change the number of binding sites or their K_D for [^{3}H]GA_4 (Table 1).

Specificity of the GA-binding Site:—

The *in vitro* competition studies while providing biological significance to the binding data are useful in another way. Since these studies are done under conditions of little or no metabolism and the concentration of GA supplied reflects the GA concentration at the site of action, they provide much more direct informaton on structure - activity relationship than do the bioassays and the relative affinity (I_{50}) values can be used to deduce some of the structural features of the GA-binding site (see Yalpani and Srivastava, 1985).

Thus, the binding protein in cucumber shows a structural specificity for γ-lactonic C-19 GAs with a C3-hydroxyl and a C6-carboxyl group. Additional hydroxylations of C16 in the D ring and C13 and C12 in the C ring impede binding, whereas changes in the hydroxylation pattern of the A ring either curtail binding affinity or completely eliminate it. The environment of the active site in the vicinity of C18 and the 1α-, 2α- and β-positions appears to be strongly hydrophobic, whereas that in the vicinity of 3β-OH, the γ-lactone ring and the C6-carboxyl is strongly hydrophilic. For the binding protein in Dwarf pea, GA_1, and GA_3 displaced the radiolabeled ligand to a much greater extent than equivalent amounts of GA_4 or GA_7 (unpublished data) which indicates that the amino acid composition of the receptor protein in pea in the vicinity of 13-OH is different from that in cucumber and is strongly hydrophilic. These competition data provide information on which parts of the GA molecule can be used for covalent attachment to an affinity matrix or for photoaffinity labelling.

Purification of the Cytosolic Receptors:—

Purification protocols using 100,000 xg cytosol from Dwarf pea and open column size exclusion and ion-exchange chromatography have yielded several fractions which show specific and exchangeable binding of [^{3}H]GA_4 (Liu, 1988). Further discrimination between these fractions was possible by using competing and noncompeting analogs such as GA_1, GA_4ME, 3-epi-GA_4. On that basis the fraction that showed the most enriched receptor-type binding (about 18 fold enrichment) had a molecular weight range of 40-100 kdaltons and eluted between 0.15-0.22 M KCl (Table 2). Some enrichment in number of binding sites mg^{-1} soluble protein was seen. Some purification (about 4x) of the binding protein was also obtained in cucumber after fractionation with ammonium sulfate and use of ion-exchange and hydroxylapatite columns (Yalpani and Srivastava, 1987). Further purification by these procedures is not possible because of the very small amounts and general lability of the protein and must await development of large scale purification protocols using differential DNA and phosphocellulose chromatography (e.g., Coty *et al.*, 1978) or alternatively, suitably derivatized affinity columns. The *in vitro* competition studies (Yalpani and Srivastava, 1985) have shown that C16 methylene group in D ring is not important for binding to the receptor protein and may offer a suitable site for covalent attachment to an affinity matrix or for photoaffinity labelling.

Table 2. Purification of [^{3}H] GA_4 Binding Protein in Dwarf Pea.[a]

FRACTION	Specific binding (pmol.mg^{-1} protein)	Purification	n[b] (pmol.mg^{-1} protein)
100,000 xg cytosol	0.006	1.0	0.28
$(NH_4)_2SO_4$ pptd. desalted protein	0.014	2.3	0.66
Fraction C (from Sephacryl S-200)	0.054	9.0	—
0.15-0.22 M KCl fraction from DE-32	0.11	18.3	—

[a]Data from Liu (1988).

[b]n calculated from Scatchard plots using pooled fractions from several extractions.

Use of Monoclonal Antibodies:—

In recent years MacMillan and his group have developed several monoclonal antibodies (McAb) against [^{3}H]GA_1 and [^{3}H]GA_4 linked to a conjugated protein (limpet hemocyanin) at C3 via hemisuccinate (Knox *et al*, 1987, 1988). While the bond between the succinate and hemocyanin is an amide bond, that between the C3-OH and succinate is an ester link and could be hydrolysed by esterases in living tissue (see Formstecher and Lustenberger, 1987).

These antibodies are reported to recognize various GA epitopes and, as expected, changes to ring A structure have less effect on binding than changes to D, C or B rings, an opposite of what would be predicted for the gibberellin receptor. Antibodies that specifically recognize rings A and B, especially the hydroxylation pattern of ring A, the C4-19γ lactone, and the 7-COOH are much more likely to lead to the GA receptor. In this connection, a report by Hooley (1988) that McAb 182 specifically recognizes rings A and B of GA_4 is of interest. Anti-idiotypic antibodies raised against McAb 182 inhibited the GA_4-induced production of α-amylase by oat protoplasts and could be used as an affinity matrix for purification of the receptor.

[^{3}H]GA_4 binding by isolated nuclei:—

In a recent study, nuclei from the target region (top 1 cm) of cucumber hypocotyls were extracted and purified on a Percoll gradient. The nuclei from between 40 to 60% interface showed specific binding of [^{3}H]GA_4 which was disrupted by heat (Sechley and Srivastava, 1990). In these experiments the concentration of [^{3}H]GA_4 was kept low to 10 nM to detect only high affinity binding sites. While these data do not exclude that [^{3}H]GA_4 was bound to nonprotein fractions in nuclei, by analogy with previous work it appears that receptor protein is present in the nuclei.

GA-Induced Transcription by Isolated Nuclei:—

In one of the earliest papers on the subject Johri and Varner (1968) demonstrated that nuclei isolated from target regions of Dwarf pea epicotyls were capable of *in vitro* transcription and that the RNA transcripts synthesized were markedly different between the controls (without GA_3) and those synthesized in the presence of GA_3. In modern terms, the transcripts produced under GA_3 were richer in mRNA and poorer in ribosomal and transfer RNA and further there was a qualitative difference between the mRNA transcripts of the GA_3 treated - vs control nuclei. These authors also noted that for this transcriptional effect to be seen it was important that the nuclei be isolated in a medium that contained GA_3 which implied the loss of a soluble GA_3-binding factor during nuclear extraction.

Nuclei isolated from protoplasts of barley and oat aleurone tissue have been shown to carry on *in vitro* transcription (Jacobsen and Beach, 1985; Zwar and Hooley, 1986). The transcripts were mostly run on transcripts, and, as expected, those obtained from nuclei which came from protoplasts kept in GA_3 were rich in mRNA for α-amylase and had reduced levels of rRNA.

Sechley and Srivastava (1990) used nuclei from the top 1 cm of cucumber hypocotyls for *in vitro* transcription studies. The extraction protocol did not include exogenous GA_4. While all nuclei showed a baseline transcription, different populations of nuclei varied in their response to added GA_4. Nuclei from the 40-60% Percoll interface showed the highest increase in transcriptional activity, about 30-35% above baseline, to 100 nM GA_4, those from the bottom and 80% Percoll interface showed no increase, while the nuclei from the top-40% Percoll interface showed an intermediate response (Fig. 3). The GA_4-induced transcription was susceptible to α-amanitin and could be improved considerably if nuclei were washed not just 2, but 3 to 5 times. It was suspected that some factor in the nuclei was inhibiting GA_4-induced transcription but not the transcription in control nuclei. Addition of nuclei washed 2 x to the assay mixture containing nuclei washed 5 x reduced the GA_4-enhanced transcription; this reduction was not obtained if 2 x washed nuclei were heat-denatured prior to their addition. These data suggest the presence of a soluble protein in nuclei which can be washed out and which inhibits the [^{3}H]GA_4-induced transcription.

Sechley and Srivastava (1990) also studied the effect of adding the enriched binding protein fraction from the 100,000 xg cytosol on transcriptional activity of isolated nuclei. Addition of the cytosolic protein alone, up to 20 μg, increased the transcriptional activity by about 40 to 50% over controls with no protein, but it did not significantly increase the GA_4-induced transcriptional activity. A similar lack of increase in steroid-induced transcriptional activity of isolated nuclei on addition of cytosolic protein is known (Buller *et al.*, 1976).

While these data need to be substantiated by other experiments, they do suggest that nuclei from the top 1 cm of cucumber hypocotyl differ in their response to exogenous GA_4, those from the 40-60% Percoll interface are specially sensitive to exogenous GA_4 and this GA_4-induced transcription is sensitive to α-amanitin. These nuclei also show specific exchangeable binding of [^{3}H]GA_4. The addition of enriched 100,000 xg cytosolic protein to *in vitro* transcription

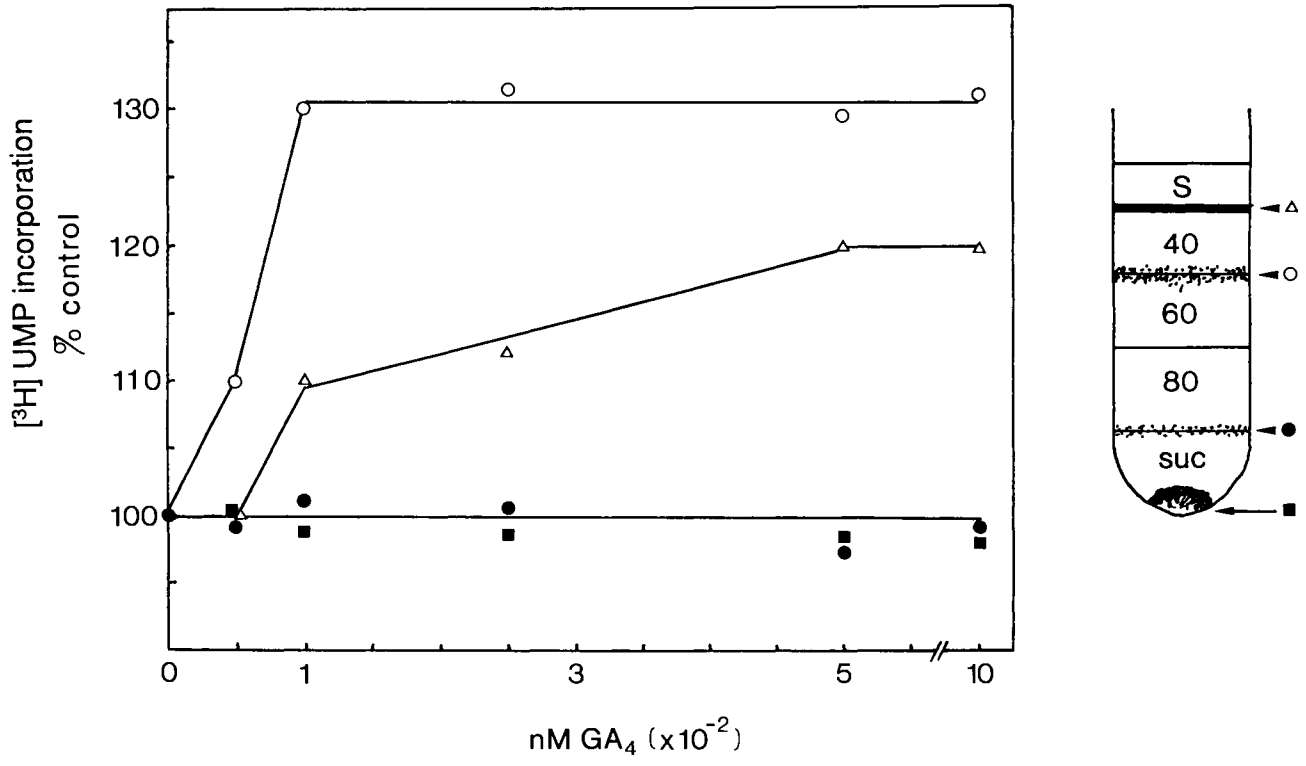

Fig. 3. GA_4-induced transcription by isolated nuclei from target region (top 1 cm) of cucumber hypocotyl. Figure on right shows the Percoll gradient and nuclear populations used in transcription assay. Control = no added GA_4. Nuclei from 80% and bottom of Percoll gradient showed no increased transcription at any GA_4 concentraton used. (From Sechley and Srivastava, 1990).

cocktail does not significantly affect the GA_4-induced transcription by these nuclei. Finally, the nuclei appear to have a soluble protein which inhibits [^{3}H]GA_4-induced transcription and which can be removed by repeated washings.

Attempts to separate the GA_4-specific poly A - mRNA fractions from isolated nuclei of cucumber hypocotyls and to translate them *in vitro* have not been successful to date. If successful, they may provide new biochemical markers, in addition to those from the aleurone system, for early evidence of GA-action.

GA-insensitive mutants:—

GA-insensitive mutants offer a unique tool to study the mechanism of gibberellin action. Several GA-insensitive dwarf mutants are known, for example *Rht3* mutants in wheat (Gale and Marshall, 1973; Gale and Youssefian, 1985), *D8* mutant in maize (Phinney, 1961; Fujioka *et al.*, 1988), *lk* mutant in pea (Reid, 1987), *gai* mutant in *Arabidopsis* (Koornneef et al., 1985).

The *Rht3* mutant in wheat is a single gene dominant mutant and the degree of nonresponsiveness to exogenous GA_3 is dose dependent on the number of alleles present (Gale and Marshall, 1975; Fick and Qualset, 1975). The mutant shows little or no shoot elongation or α-amylase production by aleurone tissue in response to exogenous GA_3 (Gale and Marshall, 1973, 1975), but is reported to be similar to the tall *rht3* genotype in terms of GA metabolism (Stoddart, 1984) and some other metabolic parameters (Ho *et al.*, 1981), and its endogenous GA content is equivalent to or higher than in the *rht3* genotype (Lenton *et al.*, 1987). It has been suggested that the insensitivity of the *Rht3* mutant to GA may be due to the production of a GA antagonist which acts upon the "active site" of gibberellin action (Gale and Marshall, 1975; see also Lenton *et al.*, 1987) or that the *Rht3* genotype may be a receptor mutant (Ho *et al.*, 1981; see also MacMillan, 1987). The *D8* mutant in maize is also a single gene dominant mutant (Phinney, 1961). Recently, it was reported to contain the same pattern of endogenous GAs as the normal variety and it was suggested that "...*Dwarf-8* may be a GA-receptor mutant or a mutant that controls a product downstream from the binding of the bioactive GA to a receptor" (Fujioka *et al.*, 1988).

Srivastava *et al.*, (1990) used protoplasts from aleurone tissue of Ramona 50, a normal tall variety of wheat, and D6899, a dwarf variety carrying the *Rht3* gene. The protoplasts were lysed, mixed in a 1:1 proportion and given exogenous GA_3 to see if the inability of D6899 to produce α-amylase could be overcome in the presence of receptor protein from Ramona 50. The lysed protoplasts responded similarly to intact protoplasts and those from Ramona produced substantially more α-amylase in the presence of GA_3 than the controls, whereas those from D6899 did not. A 1:1 mixture of lysed Ramona and D6899 protoplasts gave an α-amylase response that was intermediate between the two suggesting that the two genotypes were behaving independently (Table 3). The possibility that there was a post-transcriptional block in D6899 such that α-amylase transcripts could not be translated was discarded as it could be shown, using α-amylase cDNA probes, that GA_3-induced mRNA transcripts for α-amylases were produced in Ramona 50 but not in D6899 protoplasts. In further work it was shown that both Ramona 50 and D6899 protoplasts bound [^{3}H]GA_1 saturably and exchangeably. No data were obtained on number (n) and affinity (K_D) of binding sites for [^{3}H]GA_1 and, hence, it cannot be excluded that D6899 is a receptor mutant; nevertheless, the data obtained so far suggest that D6899 has the normal complement of receptor protein.

An accurate explanation of GA-insensitivity in wheat varieties carrying the *Rht3* gene is not yet possible. The GA-insensitivity may be due to a mutated inhibitor protein which, in contrast to the situation in the normal tall variety, is not dislodged from the regulatory element by the activated receptor. Alternatively, it is possible that the inactivating protein binds to the receptor and prevents its activation instead of binding to HRE. Several heat shock proteins, especially hsp 90, are known to associate with various kinases and steroid receptors to form inactive complexes (Moudgil, 1987; Beato, 1989). Significantly, Singh and Paleg (1984a,b) reported that preincubation of deembryonated half seeds or isolated aleurone layers of wheat varieties carrying the *Rht* genes (*Rht-1, -2, -3*) at 5°C for 20 h restored the normal response to exogenous GA_3 in terms of α-amylase production. Also, wheat plants nullisomic for chromosome 4A, which is the site for the *Rht3* gene, show normal response to exogenous GA_3 (see Gale *et al.*, 1975). These data are consistent with the hypothesis that the *Rht* gene produces a protein at room temperature which binds to the receptor and prevents its activation by the GA or alternatively prevents the activated receptor from binding to the HRE. Sechley and Srivastava (1990) noted that nuclei isolated from target regions of cucumber seedlings grown at 35°C do not show GA_4-induced mRNA transcription but those grown at 27°C do. (Although it is assumed here that the inhibitor protein binds to the HRE for α-amylase genes, or to the receptor preventing its activation by GA, the block could instead be at any of the promoter sequences of the responsive genes).

In contrast to the single gene dominant mutants that are GA-insensitive, there are single gene recessive mutants which in homozygous state behave as if they are continually saturated with GA. They are constitutively turned on and appear to have no requirement for endogenous or exogenous GA (e.g., the slender (*sln*) genotype of

Table 3. α-Amylase Production by Ramona and D6899 Protoplasts[a]

Treatment	0 h ± SD		48 h ± SD		96 h ± SD		% change from 0 to 96 h	
Ramona intact, control	1044[b]	28	1224	109	1470	110	41	—[c]
Ramona intact, +GA	1192	24	2107	158	2986	144	151	126
Ramona lysed, control	1140	10	1310	96	1668	122	46	58
Ramona lysed, +GA	1036	13	2048	188	2672	198	158	106
D6899 intact, control	1140	8	1298	72	1452	82	27	—
D6899 intact, +GA	1175	12	1420	84	1665	104	42	—
D6899 lysed, control	972	12	988	70	1085	92	12	45
D6899 lysed, +GA	998	17	1347	98	1592	112	60	58
R+D, lysed, control	1030	39	1272	118	1428	121	39	—
R+D, lysed, +GA	1088	24	1638	124	1989	168	83	98

[a]Data from Srivastava et al., (1990).

[b]α-amylase activity ml^{-1}.

[c]data from a separate experiment, — means no determination.

barley, pea; see Chandler, 1988; Lanahan and Ho, 1988; Potts *et al.*, 1985; Reid, 1987). For *sln* barley, as for pea (Potts *et al.*, 1985), it was shown recently that GA biosynthesis inhibitors, which curtailed endogenous GA levels in normal plants, had no effect on growth of slender plants and further that the half-seeds which were homozygous recessives produced α-amylase, as well as nucleases and proteases, independently of exogenous GA_3 (Lanahan and Ho, 1988; Chandler, 1988). The endogenous levels of GA were nonetheless similar in *sln* and normal half seeds (Lanahan and Ho, 1988). These data are consistent with the proposition that the inhibitor function has been lost in the slender mutant (see also Lanahan and Ho, 1988; Chandler, 1988) and that the activated receptor is locked in place on the regulatory element or enhancer sequence.

A Model for Gibberellin Action: —

Figure 4 gives a hypothetical scheme for gibberellin action which is consistent with the known data on GA biosynthesis and metabolism, GA receptor, and GA-insensitive mutants.

The main features of the scheme include the following:

1. The concentration of active GA in the target region is precisely regulated by synthetic or metabolizing enzymes (see also MacMillan, 1987) which in turn may be regulated by environmental or concentration-dependent factors.
2. The concentration of the active GA must reach a certain value to convert the receptor to an activated form.
3. The activated receptor has a high affinity for HRE and when bound to HRE is able to evoke gene expression.
4. Inhibitor proteins are present. They may be temperature-sensitive. In normal plants, the inhibitor proteins may be produced in abundance at elevated temperatures (35°C or >). In *Rht3* wheat they may be produced at room temperature. In *sln* barley they may not be produced at room temperature or not produced at all.
5. If present in abundance, inhibitor proteins block GA-induced transcription by not permitting the receptor to be activated, or, alternatively, not permitting the activated receptor to bind to HRE. (It is also possible that they act at the promoter level.)
6. An end product of GA-induced response may inhibit the continued production of active GA (feed back inhibition) or it may accelerate the inactivating enzymes, such as 2β-hydroxylase or conjugating enzymes. As a result, the level of active GA drops. The GA receptor comes off the HRE, and the inhibitor goes back on; alternatively, the inhibitor is able to bind back to receptor.
7. The activated receptor may have different affinities (K_D) for HREs of different GA-regulated genes.

A study of up- or downstream flanking regions of α-amylase genes or genes of other GA-induced products can provide useful information about the promoter sequences, the HRE and the DNA binding proteins including the GA receptor. A comparison of 5′ upstream regions of several α-Amy 2 (low pI α-amylase isozyme) genes from wheat and two α-Amy 2 type genes from barley has revealed regions of close sequence similarity up to 300 bp upstream at the start of transcription (Huttley *et al.*, 1988; Knox *et al.*, 1987; Whittier *et al.*, 1987). Regions of similarity (>600 bp) have also been found in the aligned upstream regions of different α-Amy 1 (high pI α-amylase isozyme) genes from wheat, but comparisons between α-Amy 1 and α-Amy 2 genes have shown no obvious homology (Huttley and Baulcombe 1989; for an earlier comparison of α-Amy 1, α-Amy 2 and carboxypeptidase genes, see Baulcombe *et al.*, 1986). Huttley and Baulcombe (1989) transformed oat aleurone protoplasts with promoter constructs consisting of 5′ upstream sequences of α-Amy 2/54 gene from wheat and a reporter GUS (β-glucuronidase) gene. The transformed protoplasts responded to exogenous GA_3 by producing β-glucuronidase and this response was inhibited by ABA. Transforms using promoter sequences from other genes either did not respond to GA_3 or the response was nonspecific. An analysis of 5′ deletions (from 1.9 kb to 0 b) of promoter constructs indicated that the sequences within 300 bp of the start of transcription were still sufficient to direct a high level of α-amylase production by GA_3 and its suppression by ABA (Huttley and Baulcombe, 1989).

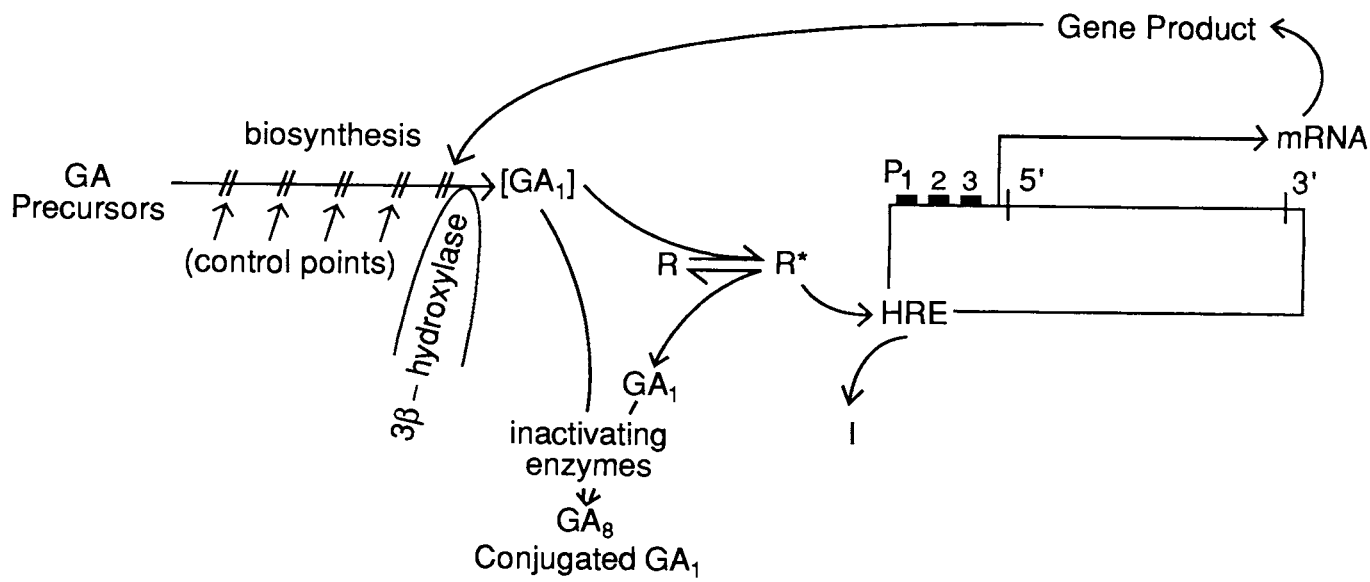

Fig. 4. A hypothetical scheme for GA action. Active GA shown is GA_1 but could be GA_4 with appropriate changes. (For various steps in biosynthesis leading from GA precursors to active GA_1 and use of synthesis mutants to confirm that scheme see MacMillan, 1987; Reid, 1987). HRE = hormone regulatory element (enhancer sequence); I = inhibitor; P_{1-3} = promoter sequences; R = GA receptor; R* = activated receptor. (The inhibitor is shown bound to HRE and released when R* binds to HRE but it could instead be bound to the R (and released when GA binds to the receptor) or to any of the promoter sequences, P_{1-3}.

In this connection it is noteworthy that Ou-Lee *et al.* (1988) reported the presence of a factor that bound to an 80 bp sequence within a 500 bp sequence upstream of a rice α-Amy 2 type gene. The factor was produced in response to GA_3 treatment in seeds or aleurone tissue (but not in leaves, roots or embroyless seeds), and did not show any measurable binding to radioactive gibberellin (the authors did not specify the gibberellin or the assay). Thus, while this factor does not appear to be the receptor protein it may be a tissue specific *trans*-factor essential for the expression of α-Amy 2 gene in rice aleurone.

Other Models of Gibberellin Action: —

There are reports of direct interaction between gibberellins and DNA (Devlin and Witham, 1983, p. 404) and several reports of changes in membrane permeability and membrane phospholipids as a result of gibberellin treatment (for earlier literature, see review by Stoddart and Venis, 1980). More recently, Singh and Paleg (1984c, 1986) reported changes in phosphatidyl ethanolamine and phosphatidyl choline in aleurone tissue of *Rht* wheat varieties given a cold temperature preincubation which earlier had been shown to remove the GA-insensitivity. The changes in phospholipids as well as GA insensitivity could be duplicated by preincubating aleurone layers for 4 h in 342 μM IAA. Singh and Paleg (1984c, 1986) suggested that GA receptors are membrane-bound phospholipids and that the *Rht* mutants have an aberrant phospholipid/fatty acid composition or metabolism. Hooley (1988) using fluorescent antibodies reported that the GA-receptor was localized on the plasmalemma of oat protoplasts.

If a plasmalemma location for gibberellin receptor is confirmed it becomes necessary to postulate the existence of a secondary messenger which migrates to the nucleus to elicit the response of differential gene regulation. Gibberellin responses, which do not require gene expression, may indeed be mediated by a plasmalemma-based receptor, but we are not aware of those responses. Unlike auxin, gibberellins are not known to have a polar transport (Jacobs *et al.*, 1988), nor have they been implicated in proton extrusion (Stuart and Jones, 1978).

Summary and Conclusions: —

There is strong circumstantial evidence for the existence of gibberellin receptors, evidence which comes from regulation of gene expression in aleurone tissue and in stem elongation and the structural specificity of the gibberellin molecule required for biological activity (see Srivastava, 1987). In comparison to steroid and thyroid hormone receptors, however, our information on gibberellin receptors is still very fragmentary.

There is evidence of *in vivo* as well as *in vitro* binding of tritiated GAs to cytosolic proteins in several plant tissues, especially cucumber hypocotyls and Dwarf and Tall pea. This binding satisfies the criteria of saturability, exchangeability, high affinity and biological specificity. There is evidence of similar binding in aleurone tissues of barley and wheat and isolated nuclei from cucumber hypocotyls. In the few cases where n and K_D have been determined, they are in the ranges expected from endogenous gibberellin levels and concentrations required for maximal biological response. In purification protocols binding occurs to several protein fractions some of which may be enzymatic proteins whereas others likely are more enriched receptor protein fractions.

There is as yet no good evidence for a structural change in the cytosolic receptor on binding to GA (but see Keith and Rappaport, 1987), or for its migration to nucleus, nor are there any studies correlating the activated receptor in the nucleus with the cytosolic receptor. For further progress to be made in gibberellin receptor work, it is essential to develop purification protocols using affinity matrices as well as photoaffinity labelling. In this connection the development of an anti-idiotypic antibody against GA_4 (Hooley, 1988) may provide suitable matrices for receptor purification, and the C_{16} methylene group may be useful in developing an affinity matrix.

Purified receptor preparations are essential for a more precise determination of [^{3}H]GA-binding characteristics than has been possible so far. Information on these characteristics is also essential for determination of changes in number or affinity of receptor protein in different tissues and at different developmental stages, and whether or not there indeed are GA-receptor mutants. The purified receptor could lead to cloning of the receptor gene and a study of the molecular interactions between gibberellin and the receptor, between the activated receptor and the regulatory sequences, and evolutionary changes in the receptor molecule.

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APPENDIX I

The methodology for sample preparation is given in scheme 1.

Several binding assays were used (For a detailed discussion of the various ligand binding assays and binding kinetics, see Venis, 1985). Equilibrium dialysis is the standard method for determining binding of ligands to macromolecules. Binding of [^{3}H]GAs to intact aleurone layers (Keith *et al.*, 1980), aleurone protoplasts (Srivastava *et al*, 1990), or cut slices of pea and cucumber stems (Keith and Srivastava, 1980; Keith *et al.*, 1981) utilizes the same principle. But it is a relatively slow method at 0°C and unsuitable for processing a large number of samples. For *in vitro* studies using cytosolic protein fractions the time factor becomes even more critical because the receptor proteins are labile and easily degraded under extraction conditions.

A typical binding assay consists of incubating the cytosol or protein fractions in a known concentration of the radioactive ligand without or with a 100-1000 fold excess of nonradioactive ligand. Other competing or noncompeting ligands may also be used (see Yalpani *et al.*, 1987). After incubation (1-2 h at 0°C), it is necessary to have a rapid filtration method to separate the tritiated GA bound to the protein fraction from that which is free in the incubation mixture. Several methodologies are available but they all suffer to a greater or lesser extent from the problem of dissociation of the radioactive ligand from the protein under nonequilibrium conditions. Gel filtration can be used (see Stoddart and Rappaport, 1974;

Keith *et al.*, 1981; Keith and Rappaport, 1987; Lashbrook *et al.*, 1987) but it is relatively slow. (The lack of $[^3H]GA_1$ binding to macromolecular fractions reported in earlier studies (see Kende and Gardner, 1976) may have been due to dissociation of the tritiated GA from the binding sites.) Other methods include use of dextran-coated charcoal (DCC) which absorbs the free ligand, the DCC can then be centrifuged out leaving the ligand bound to the macromolecule in the supernatant. The methodology is common for much of the work on steroid and thyroid hormones, and also for the soluble auxin receptors (see van Telgen *et al.*, 1986). Its use for gibberellins, however, was found to be unsatisfactory (see Keith *et al.*, 1982). A saturated solution of cold ammonium sulfate can be used to precipitate the protein and with it the bound ligand. The supernatant can be discarded (see Venis, 1985). It was used successfully to show $[^3H]GA_1$ and $[^3H]GA_4$ binding to 100,000 xg cytosol from Dwarf pea (Liu, 1988). Compared to the DEAE-filter paper assay it gave a lower numerical value of K_D for $[^3H]GA_4$ and slightly higher number of binding sites.

For gibberellin work we developed a DEAE-filter paper assay (for details see Keith *et al.*, 1982; Yalpani *et al.*, 1987). This assay is fast, reproducible, suitable for a large number of samples and thus could be used for kinetic studies. However, it requires washing with aqueous buffer and hence was suitable for $[^3H]GA_4$, but not $[^3H]GA_1$. The reason lies in the partition coefficients of these GAs in aqueous buffers and the fact that specific binding to the putative receptor is noncovalent, exchangeable, and is disrupted under nonequilibrium conditions.

SCHEME 1. EXTRACTION PROTOCOL

DWARF PEA PISUM SATIVUM L. CV. PROGRESS NO. 9
(dark grown 8-9 days, 25°C)

HARVEST TOP 1 CM OF EPICOTYL

HOMOGENIZE (100 mM phosphate buffer) on ice
+ 1 mM EDTA + 50 μM PMSF or
+ 5 mM DTT) 2-3°C

FILTER & CENTRIFUGE (100 000 g 1.5 h)

SUPERNATANT
(+ solid $(NH_4)_2SO_4$ to 60% conc.)

PELLET (Tris HCl buffer)
(desalt in Sephadex G-25 or -50 column)

PARTLY PURIFIED PROTEIN (lyophilize, if necessary)