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Auxin receptors

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1.1   Introduction

It is generally accepted that growth and development of higher plants are somehow controlled by low molecular weight compounds, called growth substances or plant hormones. Up to now five classes of growth substances have been identified, namely auxins, cytokinins, gibberellins, abscissins and ethylene. However, we cannot exclude that other classes of growth substances will be discovered in the near future. For example, evidence is accumulating that endogenous steroids may play a role in both vegetative and generative development of higher plants (Geuns, 1982).

Growth substances are produced to a greater or lesser extent in all parts of the plant and circulate via the vascular system and ground-tissue through the whole plant body. Synthesis, degradation, inactivation, interconversion, transport of the growth substances and the sensitivity of tissues to them are not only influenced by internal factors (state of tissue differentiation, age of tissues, etc.) but also by external ones (light, temperature, gravity, mechanical stress, etc.). In general, growth substances have a wide action spectrum and most processes appear to be controlled by more than one class of growth substances. The picture is very complicated and in spite of more than 50 years of research we still do not know the exact role of growth substances in overall and local morphogenesis (Trewavas, 1981, 1982; Hanson & Trewavas, 1982). Moreover, the literature comprises numerous reports describing effects of growth substances, but virtually nothing is known about their molecular mechanism of primary action. However, increasing use of modern concepts and techniques from cell biology, biochemistry and molecular genetics during the past decade gives us hope that in the near future major progress will be made in the unravelling of the molecular mecha-

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nism of action of growth substances. The introduction of the receptor
concept and the use of modern techniques to identify receptors for plant
growth substances are examples. This chapter deals with auxin receptors,
or merely with auxin-binding sites, since no receptor function has yet
been identified unambiguously.

In addition to the naturally occurring auxins (Fig. 1.1), many synthetic
auxin analogues have been synthesized and structure–activity rules have
been formulated (Katekar, 1979; Kaethner, 1977; Farrimond, Elliott
& Clack, 1978). It is general practice to call a compound an auxin if
it stimulates growth by cell extension in particular bioassays, such
as coleoptile and stem segments from etiolated monocotyledonous
seedlings (mostly grasses such as *Avena sativa, Zea mays*) and stem-
internode segments from etiolated dicotyledonous seedlings (mostly
leguminous species such as *Pisum sativum, Glycine max, Phaseolus vul-
garis*) (Larsen, 1961). A substance is called an anti-auxin if it reversibly
inhibits growth induced by an auxin (Housley, 1961).

Major production centres of endogenous auxins are the shoot apex,
leaves, developing seeds and fruits. Auxin produced by these tissues
is translocated through the whole plant via the vascular system and
unidirectionally (polar transport) via ground-tissue. Auxins appear
somehow to control elongation and branching of shoots and roots, forma-
tion and activity of cambia, cell division and formation of adventitious
shoots and roots, flower and fruit development, development and abscis-
sion of leaves, etc.

From all this, we must conclude that auxin receptors may be present
in a great variety of tissues along with receptors for other growth sub-

Fig. 1.1. A. Structure of the naturally occurring auxin indole-3-acetic
acid (IAA) and three synthetic auxins, indole-3-butyric acid (IBA),
naphthalene-1-acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid
(2,4-D).
B. Structure of the phytotoxin fusicoccin (FC).
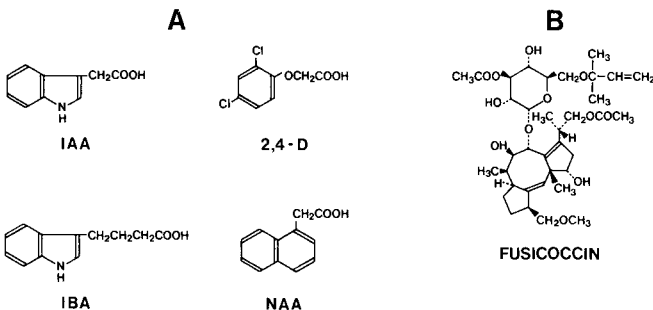
stances. To study exclusively auxin receptors, the experimental systems most widely used are those in which auxin seems to be a major limiting factor, i.e., the classical auxin bioassays. In this chapter we will try to evaluate critically the present state of auxin-receptor research. Previous reviews on growth-substance receptors are those by Kende & Gardner (1976), Venis (1977a), Stoddart & Venis (1980) and Rubery (1981), whereas short evaluations have been given by Libbenga (1978), Lamb (1978), Bogers & Libbenga (1981) and Venis (1981). For general information about plant growth substances, including auxins, the reader is referred to Letham, Goodwin & Higgins (1978).

1.2 Membrane-bound binding sites

1.2.1 Characterization of auxin-binding sites

During the last ten years auxin-binding studies have been performed with membrane preparations from various tissues and species. We have listed the main binding characteristics in Table 1.1. As can be seen from this table, membranes from maize coleoptiles have been studied most extensively and, consequently, this system will be discussed first.

(1) *Zea mays* coleoptiles

The first report on high-affinity auxin-binding sites was that by Hertel, Thomson & Russo (1972). They successfully introduced the centrifugation method for separation of bound and free ligand, experimental determinations of non-specific binding and Scatchard analysis (see section 1.4.4). Their report initiated a number of very interesting auxin-binding studies in particular on coleoptile membranes.

In discussing these studies we shall confine ourselves to the major reports by Ray, Dohrmann & Hertel (1977a,b), Ray (1977), Batt, Wilkins & Venis (1976), Batt & Venis (1976), Murphy (1980a), and Dohrmann, Hertel & Kowalik (1978). Solubilized binding sites will be treated separately in section 1.2.2.

Ray *et al*. (1977a), using crude microsomal preparations, demonstrated high-affinity, low-capacity binding for NAA (K_d, 0°C = 5–7 × 10⁻⁷ M, n = 30–50 pmol/g fresh wt) from straight Scatchard plots obtained after correcting for experimentally determined non-specific binding. They found that rapid binding (half exchange time for naphthylacetic acid (NAA) < 15 min) occurs at 0°C and shows a sharp optimum at pH 5.5. The binding site is rapidly inactivated at temperatures above about 30°C and is sensitive to pronase, in particular after pretreatment of the membrane preparations with phospholipase.

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Table 1.1. *Characteristics of membrane-bound binding sites*

Material	Fraction[a]	Ligand	Binding sites K_d (μM)	conc.[b]	Specificity	pH	Temp. (°C)	Time[c]	Reference
Zea									
coleoptiles	CM	IAA	3–4	30	–	6	0	<30	Hertel et al. 1972
	CM	IAA	1.7	51	+	5.5[d]	0	<15	Batt et al.[e] 1976
	CM	IAA	5.8	96	+	5.5[d]	0	<15	Normand et al. 1975, 1976
	CM	IAA	—	—	+[g]	6	0	5	Hertel et al. 1972
	CM	NAA	1–2	40	+	6	0	<30	Ray et al. 1977a,b
	CM	NAA	0.5–0.7	30–50	—	5.5[d]	0	<15	Moloney et al. 1981
	CM	NAA	0.5	38[h]	+	5.5	20	10	Normand et al. 1977
	CM	NAA	1.9	620[i]	+	6	4	5	Batt et al.[e] 1976
	CM	NAA	0.15	38	—	5.5[d]	0	<15	Murphy 1980a
	CM	NAA	1.6	96	—	5.5[d]	0	<15	Batt et al. 1976[f]
	CM	NAA	0.15	100[i]	—	5.5	0	<15	Murphy 1980a
	H	NAA	1.16	32	—	5.5[d]	0	<15	Batt et al. 1976[f]
	H	NAA	0.5	—	+	5.5	0	<15	Murphy 1980a
	L	NAA	0.39	24	—	5.5[d]	0	<15	Normand et al. 1977
	L	NAA	0.44	—	—	5.5	0	<15	Ray 1977
	ER	NAA	1.7	15	+	6	0	5	Dohrmann et al. 1978
	ER	NAA	0.4	40	+[k]	5.5[d]	0	<30	Normand et al. 1977
	TP	NAA	1.3	20	—[k]	5.5	0	<30	Walton et al. 1981
	PM	2,4-D	5	40	—	5.5	0	<30	Moloney et al. 1981
mesocotyls	CM	NAA	0.75	52	—	6	4	5	Normand et al. 1977
	ER	NAA	0.2	1.9[i]	—	5.5[d]	—	—	Walton et al. 1981
roots	PM	NAA	0.8	16	—	5.5	20	10	Moloney et al. 1981
	PM	NAA	1.2	19	+[k]	5.5	20	10	Moloney et al. 1981

Tissue									Reference
Avena									
roots	CM	IAA	12	346	—	5.5^d	37	30	Bhattacharyya et al. 1978
roots	CM	IAA	52	989	—	5.5^d	37	30	Bhattacharyya et al. 1978
	CM	IAA	0.68	15^i	—	5.5	0	<15	Bhattacharyya et al. 1982
coleoptiles	CM	IAA	7	57^i	—	5.5	0	<15	Bhattacharyya et al. 1982
	CM	IAA	0.2	12^i	—	5.5	0	<15	Bhattacharyya et al. 1982
	CM	IAA	7	81^i	—	5.5	0	<15	Bhattacharyya et al. 1982
Glycine									
hypocotyls	PM	IAA	—	—	—	6	4^d	i	Williamson et al. 1977
Phaseolus									
hypocotyls	PM	IAA	—	—	—	6.5^l	4	20	Kasamo et al. 1976
Pisum									
cotyledonary buds	CM	IAA	0.1	—	—	6.5^d	25^d	30	Jablonović et al. 1974
epicotyls	PM	IAA	1^m	0.1^i	—	$6{-}6.5^d$	4	f	Döllstädt et al. 1976
roots	PM	IAA	1^m	0.1^i	—	$6{-}6.5^d$	4	f	Döllstädt et al. 1976
epicotyls	PM	MCPA	1^m	0.1^i	—	6–6.5	4	f	Döllstädt et al. 1976
roots	PM	MCPA	1^m	0.1^i	—	6–6.5	4	f	Döllstädt et al. 1976
Cucurbita									
hypocotyls	PM	IAA	1.5	20	+	5^d	2–4	<15	Jacobs et al. 1978
Helianthus									
tuber explants	CM	IAA	1	22^h	—	5.5	4	<30	Trewavas 1980
Nicotiana									
callus	CM	NAA	0.3	58	+	5^d	36^d	30^d	Vreugdenhil et al. 1979
stem pith	CM	NAA	0.3	32	+	5^d	36^d	30^d	Vreugdenhil et al. 1979
leaves	CM	NAA	0.5	18	—	5	36	30	Vreugdenhil et al. 1980
leaf protopl.	CM	NAA	0.4	36	—	5	36	30	Vreugdenhil et al. 1980
cell suspension	CM	NAA	0.2	$—^n$	—	—	36	30	Vreugdenhil et al. 1981

Table 1.1 (*Contd.*)

Material	Fraction[a]	Ligand	Binding sites		Specificity	pH	Temp. (°C)	Time[c]	Reference
			$K_d(\mu M)$	conc.[b]					
Cucumis young fruits	CM	NAA	10–20	1250	+	3.75^d	0	10	Narayanan *et al.* 1981a
Fragaria receptacles of developing fruits	120K	NAA	1.1	100	+[o]	4^d	0	10	Narayanan *et al.* 1981b

See Appendix 1 for definitions of abbreviations.
[a] CM = crude membrane, PM = plasma membrane, ER = endoplasmic reticulum, TP = tonoplast, H = heavy band, L = light band.
[b] Concentration expressed as pmol/g fresh weight, unless otherwise indicated.
[c] Time expressed in minutes or: i = immediate, f = fast.
[d] Optimal conditions determined.
[e] Batt, Wilkins & Venis (1976).
[f] Batt & Venis (1976).
[g] No K_d determined.
[h] Not given by authors but determined by us from their Scatchard plots.
[i] pmol per mg protein.
[j] Maximum value.
[k] Related to elongation test or curvature test.
[l] Optimum binding at pH 8.
[m] Not from Scatchard plots.
[n] Varying, see text.
[o] Related to receptacle enlargement.

The specificity of the site was described by Ray *et al.* (1977*b*), who determined K_d values for many auxins, auxin analogues (agonists and antagonists) and non-auxins. These K_d values were compared with the C_{50} values (the concentration at which a half-maximal effect is reached) obtained from classical maize coleoptile elongation tests (see section 4). They found large discrepancies between the K_d and C_{50} values, especially for the phenoxyacetic acids ($K_d/C_{50} \gg 1$, indicating a higher efficiency than would be expected from binding studies) and for phenyl derivatives ($K_d/C_{50} \ll 1$).

This is no surprise because:

> Binding of different analogues do not necessarily all lead to the same maximal (quantitative) response (see e.g., Ariens, Beld, Rodrigues de Miranda & Simonis, 1979).

> The analogues tested are possibly subject to differences in *in vivo* uptake (Evans & Hokanson, 1969), or in sequestration and metabolic alteration (Klämbt, 1961).

> Ray *et al.* (1977*b*) found that the C_{50} values may depend upon whether growth is measured after a relatively short or after a long period. A reasonable explanation for this phenomenon is that different, unsynchronized processes, initiated by auxin application, are treated as one process, i.e., growth.

> The binding experiments were performed at 0°C while elongation tests are performed at higher temperatures (23°C).

> The elongation of maize coleoptiles may not be affected by the binding site under consideration.

The only conclusion that can be drawn from the experiments of Ray *et al.* (1977*b*) is that, in general, auxins and anti-auxins (those compounds that inhibit elongation reversibly) bind to the same binding site competitively while non-auxins do not, or do so with very low affinity. In this respect the binding sites can be considered as specific.

In a previous report Ray, *et al.* (1977*a*) had already shown that the supernatant from microsome pellets contains a factor that lowers the affinity of the binding sites for NAA but does not affect their number. This so-called supernatant factor (SF) was later identified by Venis & Watson (1978) as a mixture of 6-methoxy-2-benzoxazolinone (MBOA) and 6,7-dimethyl-2-benzoxazolinone (DMBOA). Ray *et al.* (1977*b*) tested the influence of crude SF on the K_d values of most analogues studied. They found that SF increases the K_d values for some analogues (e.g., 1-NAA, 2-NAA, 3-indole-3′-propionic acid (IPA)), whereas K_d values for some other compounds (e.g., 2,4-dichlorophenoxyacetic acid

(2,4-D), 3,4,5-triiodobenzoic acid (3,4,5-TIBA) and 2-naphthoxyacetic acid (2-NOA)) are decreased. However, these effects of SF did not generally improve the correlation between K_d and C_{50} values.

Of course we cannot conclude from these experiments that SF is an endogenous modifier of binding affinity of auxin receptors. SF is found only in a limited number of grasses and only after the cells have been damaged (Venis & Watson, 1978 and references therein). Cross & Briggs (1979) have tested whether supernatant isolated from other species (onion, carrot, potato, apple) influences binding of NAA to binding sites in microsomes from maize coleoptiles and solubilized binding sites from the same material. They found that all species tested show some inhibitory effect, and that the largest effect is caused by supernatant isolated from maize coleoptiles.

With respect to the localization of the binding sites, Ray (1977) found that after isopycnic centrifugation of microsomes, the majority of high-affinity NAA binding sites coincide with endoplasmic reticulum (ER) markers (NADPH: cytochrome c reductase and rotenone- and antimycin-insensitive NADH-cyt-c reductase). At high Mg^{2+} concentrations, which prevent the ribosomes from being stripped off the ER, the majority of NAA binding shifts to higher densities. This agrees with a localization of the binding sites at the ER. This was quite unexpected since, with regard to the acid-growth theory of auxin action (see section 1.2.4) it was plausible to assume that auxin receptors would be localized at the plasma membrane. However, evidence indicating the presence of two classes of auxin-binding sites in maize coleoptile membranes – one localized at the ER (site 1) and another one at the plasma membrane (PM) (site 2) – is reported in Batt *et al*. (1976) and in Batt & Venis (1976). They concluded from biphasic Scatchard plots that two binding sites – one with a high affinity (site 1) and one with a lower affinity (site 2) – are present in a membrane preparation, obtained by centrifugation at 4000–38 000 **g** (Batt *et al*. 1976). Further experiments by Batt & Venis (1976) showed that a light and a heavy microsome fraction can be obtained after sucrose density gradient centrifugation. With each of these fractions straight Scatchard plots with different slopes were found. The K_d values found for the heavy and light fractions corresponded with the K_d values found for sites 1 and 2, respectively, as obtained previously by Batt *et al*. (1976) from biphasic Scatchard plots. Although it is tempting to assume the presence of two different classes of binding sites from these data, there are a few problems. In the first place, the presence of a binding site of high affinity (site 1) can indeed be concluded from the biphasic Scatchard plot shown by Batt *et al*. (1976), but their conclu-

sion that another site with lower affinity is also present was too hasty.
They analysed the plot by means of the extrapolation method without
taking into account that the flat part of the curve may have been caused
by non-specific binding. In the second place, the straight Scatchard plots
for the light and the heavy microsome fractions were obtained using
NAA in the range of 2×10^{-7} to 1×10^{-6} M. The danger of using narrow
concentration ranges is pointed out in section 1.4.2 and, indeed, when
the experiments of Batt & Venis (1976) were repeated by Murphy
(1980a), he found comparable plots in the same concentration range,
but after extending the range towards higher concentrations, the straight
lines turned into curves. He then analysed the curves with the computer
program MLP (see section 1.4.2) and concluded that the best fits are
obtained with a 3-parameter model, i.e., one high-affinity binding site
plus non-specific binding. This analysis showed that K_d values of high-
affinity binding in the light and heavy fractions are almost identical,
but that the concentrations of the binding sites are different. When we
re-examined his calculations we found something different. Using the
equation

$$\frac{B}{F} = \frac{K_1 R_1}{1 + K_1 F} + N \tag{1}$$

where B = concentration of bound hormone; F = concentration of free
hormone; K_1 = association constant; R_1 = concentration of receptor;
N = amount of non-specific binding, the values of B and B/F can be
computed and a theoretical Scatchard plot can be constructed, when
K_1, R_1 and N are known, for any hormone concentration H. We have
tried to construct such plots for binding to both the heavy and light
bands, in order to compare them with the experimental plots. We used
K_1 and R_1 as given by Murphy (1980a). As the amount of non-specific
binding was omitted in his paper, we tested a range of values for N
(by means of a short computer program based upon eq. (1). A value
for N that would give a plot that could be superimposed upon the experi-
mental plot was readily found for the heavy band, but for the light
band such a value could not be found. This would mean that either
the model used in Murphy's paper (1980a) was incorrect, or that K_1,
R_1 and/or N were calculated wrongly. We tested both a 3-parameter
model and a 4-parameter model using the programs described in section
1.4.2. We found no significant difference between the models, but we
did find a difference between K_d values for the light and the heavy bands
(respectively 2.3×10^{-6} and 8.0×10^{-6} M), thus indicating the presence
of different classes of binding sites.

Further supporting evidence for two different classes of binding sites was found by Batt *et al.* (1976), in that some compounds, which were inactive in the coleoptile elongation test, such as benzoic acid, 2,6-di-chlorobenzoic acid (2,6-D) and 2,4-dichlorobenzoic acid (2,4-D), compete with NAA for site 1 but not for site 2. This would indicate that site 1, which has the highest affinity for strong auxins like NAA, nevertheless binds some inactive compounds, whereas site 2, which has a lower affinity, is more selective in this respect.

Comparison of the different binding sites in sucrose density gradients with enzyme markers showed that the position of site 1 coincides with ER markers and that of site 2 with PM markers. Batt & Venis (1976) rightly draw attention to the fact that the use of enzyme markers to determine PM fractions is not unequivocal (see also Cross & Briggs, 1976, 1979, and references therein). However, they obtained additional evidence that site 2 is located at the PM from specific staining methods and the determination of sterol : phospholipid ratios.

Binding parameters and localization of site 1 are comparable with the NAA-binding site described by Ray and coworkers, but Ray *et al.* (1977*b*) found hardly any competition with benzoic acid, and a much lower affinity for 2,4-benzoic acid and 2,6-dichlorophenoxyacetic acid.

Finally, Dohrmann *et al.* (1978) reported that possibly three classes of binding sites are present in maize coleoptile membranes. On sucrose density gradients the major amount of the binding sites (site I) coincides with ER markers, another site (site II) is associated with acid phospha-tase, which is possibly located at the tonoplast, and a third site (site III) appears at 30–38% sucrose coinciding with a plasma membrane marker (NPA-binding). The sites show a difference in affinity for various analogues. Thus, the affinity of site I, for instance, NAA, indoleacetic acid (IAA) and the anti-auxin phenylacetic acid (PAA) is substantially higher than the affinity of site II for these analogues. Apparently PAA can be used to discriminate between site I and site II in unfractionated microsome preparations. Both site I and site II have a relatively low affinity for 2,4-D, whereas site III has a relatively high affinity for 2,4-D and a relatively low affinity for NAA and IAA. SF has no effect upon binding affinities of site II, but in the presence of SF, binding affinities of site I towards auxin analogues are altered in such a way that they resemble binding affinities of site II. In accordance with Ray (1977), the majority of the binding sites (site I), (also in the presence of SF) does not bind benzoic acid, nor does site II. In respect to site III, we must notice that the amount of 2,4-D bound was very low (75 000 cpm added, 9000 non-specifically bound and only 200 specifically) and