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AN AUXIN INDUCES THE APPEARANCE OF AUXIN-BINDING ACTIVITY IN ARTICHOKE TUBERS

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Key Word Index—*Helianthus tuberoseus*; Compositae; auxin-binding activity; cell division; auxin influx and effiux.

Abstract—Artichoke tuber tissue responds to treatment with the synthetic auxin, 2,4-D, by undergoing cell division. Specific IAA-binding activity is undetectable in crude membrane preparations made from either the intact tuber or from tissue cultures in the absence of 2,4-D. On the other hand specific IAA-binding activity is readily detectable if the tissue has first been cultured in 2,4-D. Studies of 2,4-D efflux–influx kinetics are also in agreement with this notion. Both parameters are modified and the net result is a higher level of retention of 2,4-D in this tissue if the tissue has been previously cultured in 2,4-D-containing media, suggesting the appearance of auxin-binding activity.

INTRODUCTION

Auxin-binding proteins have been detected in membrane fractions of several plant tissues [1–3] and it is thought that they could be the equivalent of the well-characterized mammalian hormone receptor proteins. In contrast root and tuber tissue, both of which respond physiologically to hormone treatment, apparently have no detectable auxin-binding activity [1] raising doubts concerning the significance of such proteins to auxin action. In this paper the situation in artichoke tuber tissue has been examined which, when cultured under sterile conditions, in darkness and in nutrient medium, responds to the synthetic auxin, 2,4-dichlorophenoxyacetic acid (2,4-D), by undergoing cell division [4]. The results indicate that auxin-binding activity of crude membrane fractions from this tissue is detectable but only after culturing tuber tissue in 2,4-D itself.

RESULTS

Effect of culturing tissue in 2,4-D on auxin-binding activity

Specific auxin-binding of crude membrane fractions from cultured artichoke tissue was measured in the way described by Hertel *et al.* [1]. This involved sedimentation of membrane fractions in 2×10^{-7} M 3-indolylacetic acid (IAA)-[^{14}C] plus or minus 10^{-4} M IAA-[^{12}C]. The term specific auxin-binding indicates labelled bound IAA whose binding is competitively inhibited by unlabelled 10^{-4} M indoleacetic acid. The term is used here with the same provisos on its meaning outlined by Ray *et al.* [2]. Each binding measurement was carried out in quadruplicate and involved the use of some 20 g sterile cultured tissue.

Fig. 1a shows the variation of specific auxin-binding of membrane fractions from tissues cultured in the presence or absence of 10^{-6} M 2,4-D for 0–3 days. Between 3 and 5 experiments were performed for each time point. The values on the graph represent the mean of all determinations and the bars the standard errors. There is clearly significant specific IAA-binding by membrane fractions isolated from tissue cultured in 2,4-D but not to tissue cultured in its absence. Although most experiments used tissue cultured in 10^{-6} M 2,4-D, two single experiments used 10^{-7} M and 10^{-5} M 2,4-D for 2 days and these have also been included. Fig. 1b shows estimates of cell numbers in the discs undergoing culture during comparable incubation conditions.

Because of complications with polyphenol formation during the homogenization of artichoke tissue, both polyvinylpyrrolidone (PVP) and mercaptoethanol (ME) were necessary components of the homogenization medium. ME, however, is known to damage the auxin-binding site in coleoptiles and, to counteract this in artichoke, the suggestion of Ray *et al.* [2] was taken up and 10^{-4} M IAA included in the homogenization medium to protect it. Subsequently the 10^{-4} M IAA was removed by two washes before auxin-binding was measured. These precautions were essential to detect specific auxin-binding in artichoke membrane fractions. The deleterious effect of ME on the artichoke-binding site was also confirmed using higher concentrations of ME (1%) in the absence of IAA. Some other similarities with binding by coleoptile membrane fractions were also noted. Specific auxin-binding could be detected at pH 5.5 but not at pH 7.4 [2, 3] and low levels of binding activity were also found in the initial 4000 g pellet of 2,4-D treated tissue although these have not been investigated further.

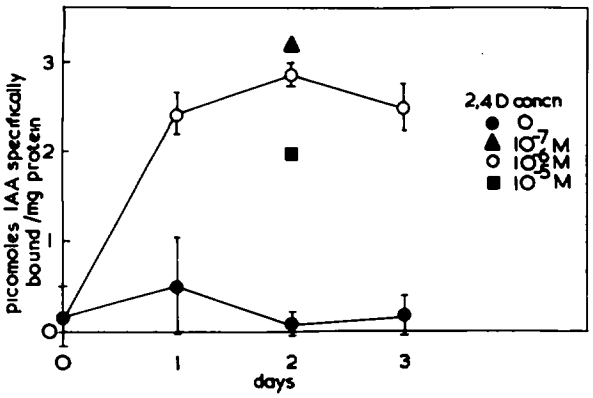

Fig. 1a. Variation with time of the specific IAA-binding capability of crude membrane fractions from artichoke tissue cultured in the presence and absence of auxin. Discs of sterile artichoke tuber tissue were prepared and cultured for 0–3 days in sucrose/mineral salts in the presence and absence of 2,4-D. At the specific times shown, the tissue was collected, homogenized and specific auxin-binding capability of the crude membrane fraction determined as described in Experimental. Binding assays were carried out in quadruplicate. Between 3 and 5 experiments were conducted for each time point. Symbols represent the means, and bars the standard errors of all determinations. Most experiments used 10^{-6} M 2,4-D in the culture medium. Two exceptions are the 2-day experiments using 10^{-7} M and 10^{-5} M 2,4-D which are also shown. ● = tissue cultured in the absence of 2,4-D; ○ = tissue cultured in 10^{-6} M 2,4-D; ▲ = tissue cultured in 10^{-7} M 2,4-D; ■ = tissue cultured in 10^{-5} M 2,4-D.

A limited attempt has also been made to determine the binding constants of the membrane fractions of 2,4-D treated tissue using a Scatchard plot (constructed as described in Ray *et al.* [2]). Fig. 2 shows the plot using membrane fractions from 3-day 2,4-D treated tissue. The number of points that could be obtained for this plot were severely limited by the quantity of sterile tissue needed for the experiment (over 50 g) and the requirement for carrying out measurements in quadruplicate. The K_d of the membrane auxin-binding sites can be calculated from the slope of the graph and is *ca* 10^{-6} M for IAA. This may be compared to that determined by Ray *et al.* [2] for coleoptiles of 5×10^{-7} M (for 1-naphthylacetic acid).

No attempt has been made to determine the stereochemical specificity of the binding site apart from demonstrating that gibberellic acid and abscisic acid have no effect on specific auxin-binding. It should be noticed, however, that the site is induced by 2,4-D and detected using IAA.

Effect of culturing artichoke tissue in 2,4-D on subsequent 2,4-D influx and efflux

Since 2,4-D induced the appearance of auxin-binding activity, the possibility that correlated changes in the influx and efflux of 2,4-D might also occur when tissue was cultured in 2,4-D was examined.

The results of efflux experiments have been summarized in Fig. 3 and Table 1. Sterile artichoke tissue, previously cultured in the presence or absence of 2,4-D, was prelabelled for 0.5 or 2 hr with 10^{-6} M 2,4-D-[2-^{14}C]. It was then transferred to culture medium containing 10^{-6} M 2,4-D-[^{12}C] and efflux measurements continued for the subsequent 3 hr. The presence or absence of 10^{-6} M 2,4-D-[^{12}C] in the chase medium did not in any way affect the efflux kinetics and thus only data in which 2,4-D included are shown here. All incubations were carried out in dim green light to avoid the complicating effect of white light on cell division [4].

Fig. 3 shows efflux curves for tissue previously cultured for 2 days in the presence and absence of

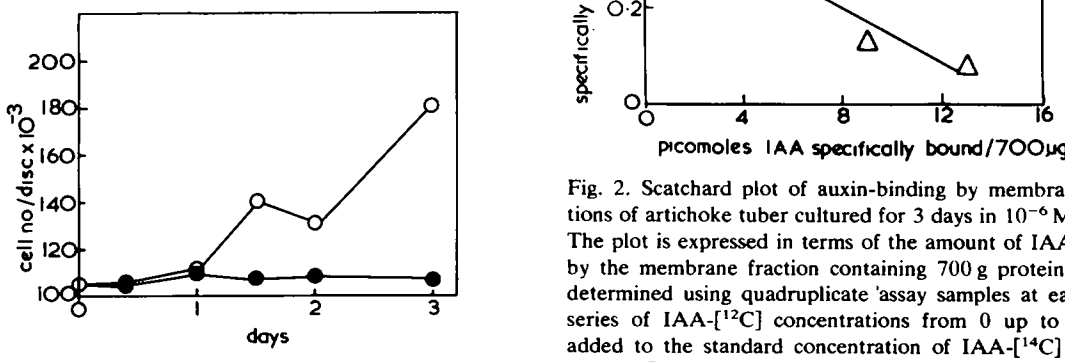

Fig. 1b. Variation with time of the cell number/disc during culturing. For determination of cell numbers at each time and treatment point three batches each containing 10 discs were incubated for 3 days in 5% chromic acid. Six cell counts using a haemocytometer were carried out on each batch and the mean ± standard error determined. Cell counts at zero time refer to tissue washed for 1 hr in distilled water to eliminate damaged and broken cells at the surface. ○ = tissue cultured in 10^{-6} M 2,4-D. ● = tissue cultured in the absence of 2,4-D.

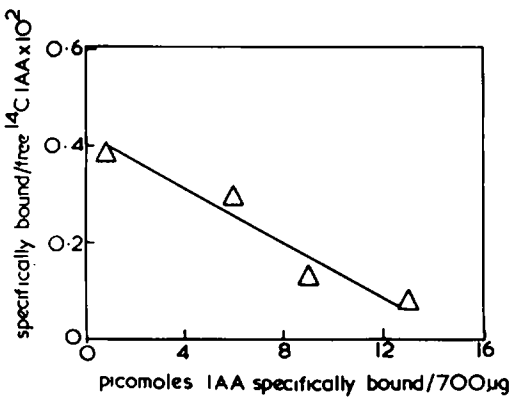

Fig. 2. Scatchard plot of auxin-binding by membrane fractions of artichoke tuber cultured for 3 days in 10^{-6} M 2,4-D. The plot is expressed in terms of the amount of IAA bound by the membrane fraction containing 700 g protein. It was determined using quadruplicate assay samples at each of a series of IAA-[^{12}C] concentrations from 0 up to 10^{-4} M added to the standard concentration of IAA-[^{14}C] (in this case 10^{-7} M). The mean values of ^{14}C in the pellets from the 10^{-4} M samples, representing non-specific binding, was subtracted from the mean for each of the lower concentrations to give the specifically bound ^{14}C for that concentration. This value was divided by the specific radioactivity of IAA in that treatment to calculate the amount of IAA specifically bound at that concentration. Bound/free IAA was calculated as ^{14}C supplied minus total ^{14}C bound, i.e. specifically and non-specifically.

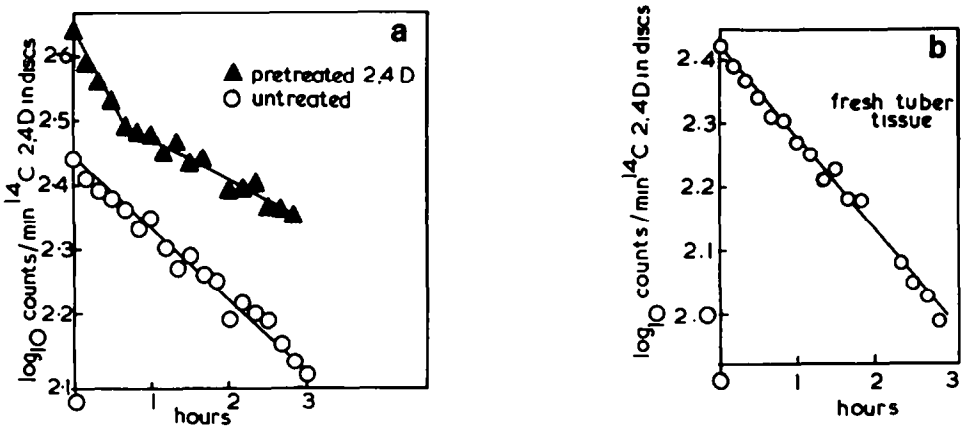

Fig. 3. Efflux of 2,4-D-[^{14}C] from tissue previously cultured in the presence or absence of 10^{-6} M 2,4-D. (a) Efflux kinetics of 2,4-D-[^{14}C] from tissue cultured for 2 days in the presence or absence of 10^{-6} M 2,4-D. (b) Efflux kinetics of 2,4-D-[^{14}C] from uncultured tuber tissue. Sterile tissue was prepared and in the case of (a) cultured for 2 days in the presence and absence of 10^{-6} M 2,4-D-[^{12}C]. Batches of 20 discs (a) and 15 discs (b) were then labelled for 0.5 hr in 10^{-6} M 2,4-D1[^{14}C], washed in sterile water and then transferred back to fresh sucrose/mineral salts containing 10^{-6} M 2,4-D-[^{12}C]. Samples of the medium were taken at 10 min intervals for the subsequent 3 hr and counted. The ordinate indicates the quantity of labelled 2,4-D remaining in the tissue at the specific time.

10^{-6} M 2,4-D and for uncultured tuber tissue. Efflux curves for 1 and 3 days were very similar to the 2-day pretreatment and they have been summarized as rate constant data in Table 1, experiment 1. Effluxed 2,4-D was chromatographically identical (*iso*-PrOH–NH_3–H_2O, 8:1:1) to pure 2,4-D-[2-^{14}C].

The efflux patterns are clearly altered by the prior culturing of the tissue in 2,4-D itself. The efflux curves from 2,4-D cultured tissues are (at least) biphasic with one compartment showing faster exchange and another much slower exchange than tissue cultured in the absence of 2,4-D. The fast exchanging pool ($t_{1/2}$ just under 1 hr) represented 25–33% of the total 2,4-D taken up in all 2,4-D treated tissues. The slower exchanging pool has a rate constant of decay *ca* half (and, therefore, $t_{1/2}$ twice as long) that of the one detectable pool in untreated tissue which may be its metabolic equivalent. Experiment 2 in Table 1 shows that this biphasic character to the efflux curve is only induced by concentrations of 2,4-D significantly higher

Table 1. Efflux of labelled 2,4-D from tissue previously cultured in the presence and absence of 2,4-D

Expt.	Preincubation Days	Preincubation 2,4-D concentration	Rate constants of efflux (hr^{-1})	
1	0	0	0.405	
	1	0	0.332	
	2	0	0.34	
	3	0	0.305	
	1	10^{-6} M	0.73 (33%)	0.18
	2	10^{-6} M	0.73 (27%)	0.20
	3	10^{-6} M	0.62 (25%)	0.17
2	2	0	0.36	
	2	10^{-7} M	0.38 (7%)	0.31
	2	$+10^{-6}$ M	0.58 (27%)	0.162
	2	$+10^{-5}$ M	0.54 (33%)	0.21

Sterile artichoke tissue was cultured for 0–3 days in the presence or absence of 2,4-D. Batches of tissue were then labelled for 0.5 hr in expt. 1 and 2 hr in expt 2 in 10^{-6} M 2,4-D-[^{14}C]. After washing with sterile water, tissue was incubated in sucrose/mineral salts 10^{-6} M 2,4-D-[^{12}C] and samples of the medium taken for counting every 10 min for the subsequent 3 hr. At the end of the incubation residual 2,4-D was estimated by extracting the discs in boiling ethanol. All incubations were carried out in dim green light. Examples of the efflux curves are shown in Figs. 3a and 3b. Rate constants were determined from natural logarithmic plots of the decay curves. Figures in parentheses in expts 1, and 2 2,4-D treatments represent total 2,4-D found in fast exchanging pool.

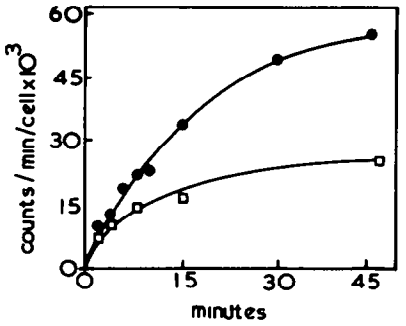

Fig. 4. Uptake of labelled 2,4-D by tissue previously cultured for 3 days in the presence and absence of 10^{-6} M 2,4-D. Sterile artichoke tissue was cultured for 3 days in sucrose/mineral salts in the presence or absence of 10^{-6} M 2,4-D. The tissue was divided into batches of 10 discs which were then labelled for the specific time period in sucrose/minerals 10^{-6} M 2,4-D-[^{14}C] before determination of their 2,4-D-[^{14}C] content. ● Tissue cultured in 10^{-6} M 2,4-D. □ Tissue cultured in the absence of 2,4-D.

than 10^{-7} M. Furthermore, it is only induced after lengthy 2,4-D treatment since tissue previously cultured in the absence of auxin receives 3.5–5 hr 2,4-D treatment with no detectable alteration in the efflux kinetics.

The effects of culturing tissue in 2,4-D on subsequent 2,4-D uptake are shown in experiments summarized in Fig. 4 and Table 2. Fig. 4 shows an experiment in which the capability of tissue, previously cultured for 3 days in the presence and absence of 2,4-D, to take up 2,4-D has been measured. Uptake characteristics for days 0–2 and day 3 are summarized in Table 2 as initial velocities of 2,4-D uptake and final equilibrium 2,4-D-[^{14}C] content. The time taken to reach an approximate equilibrium was the same in all cases, i.e. 30–45 min. Although the final equilibrium 2,4-D content/cell is much higher after prior culturing of the tissue in 2,4-D (some 2.5-fold after 3 days), this seems not to be the result in the main of a higher rate of influx as measured by initial velocity. It must therefore be mainly the result of an overall lower rate of efflux from the tissue in agreement with the data in Fig. 3 and Table 1.

DISCUSSION

There are at least two simple explanations of the auxin-binding data shown in Fig. 1. Firstly, the results can be taken at their face value as demonstrating the appearance of auxin-binding activity or binding proteins after treatment with the synthetic auxin 2,4-D. Secondly, an equal possibility is that 2,4-D inhibits the formation of substances which damage the binding site during its isolation and thus prevent its detection. The necessity for including both mercaptoethanol and polyvinylpyrrolidone in the extraction medium in order to detect the auxin-binding site has already been mentioned. Visual observation of both incubated tissue and unprotected homogenates suggests that there are higher levels of polyphenols in the untreated tissue, making this suggestion reasonable. Resolution of these two possibilities clearly requires the isolation and quantitative estimation of the levels of the binding protein itself in both 2,4-D treated and untreated tissue. Even if the binding data are accepted at their face value, it should be emphasised that the binding sites being detected here only have low affinity for auxin since we have not used concentrations of IAA for binding lower than 10^{-7} M. Other higher affinity binding sites may be presented which respond to 2,4-D pre-treatment in a totally different way. It should be noticed that Libbenga [5] reported the appearance of a high affinity soluble auxin receptor in pith tissue after IAA treatment.

Some comment must be included on the 2,4-D efflux experiments illustrated in Fig. 3 and Table 1. The efflux from 2,4-D treated tissue is quite definitely biphasic, consisting of one faster and one slower component than the single component from untreated tissue. Based on observations of ion exchange rate

Table 2. Uptake of labelled 2,4-D by tissue previously cultured in the presence or absence of auxin

Days incubation	Initial velocity of 2,4-D uptake (pmol/cell/min × 10^4)		Equilibrium 2,4-D content (pmol/cell × 10^4)	
	pretreatment		pretreatment	
	−2,4-D	+2,4-D	−2,4-D	+2,4-D
0	0.56	—	8.75	—
1	0.44	0.41	7.5	7.8
2	0.45	0.54	5.6	10.6
3	0.54	0.73	5	13.1

Sterile artichoke tissue was cultured for 0–3 days in the presence or absence of 10^{-6} M 2,4-D. Measurement of 2,4-D uptake was made by transferring to batches of 10 discs for varying time periods (2–45 min) to 10^{-6} M 2,4-D-[^{14}C] in sucrose/mineral salts with shaking. Labelled 2,4-D taken up was extracted from the washed discs with boiling ethanol. Uptake curves (an example is shown in Fig. 4) were summarized as initial velocity of 2,4-D uptake (the tangent drawn to the smoothed curve connects the points at zero time) and equilibrium 2,4-D content (the asymptote drawn to the smooth curve at 45 min).

from storage disc tissue, these two compartments in 2,4-D treated tissue may be identified with the soluble cytoplasm (fast component) and the membrane enclosed cytoplasm, rough endoplasmic reticulum, mitochondria, vacuole, etc. as the slow component. 2,4-D treatment of artichoke tissue either institutes a soluble cytoplasmic compartment from 2,4-D or, alternatively, increases the permeability of the plasma membrane to auxin. Both possibilities would explain the appearance of the fast component. An alternative suggestion that the production of more and smaller cells by 2,4-D-induced cell division would increase the surface area for 2,4-D efflux can probably be rejected for two reasons. Firstly, because the biphasic curve can be detected after 1 day's 2,4-D treatment (cell division only commenced 1–2 days after treatment, Fig. 1b) and the size of this fast efflux pool actually declined slightly from days 1–3, whereas the proportion of dividing cells continued to increase (Fig. 1b).

The slower component of the efflux curve representing probably membrane-bound cytoplasm shows a rate constant of efflux *ca* 50% that of probably the equivalent pool in untreated tissue. Since the auxin-binding activity seems to be located in this cell fraction, this reduced rate of efflux is in good agreement with the appearance of auxin-binding activity demonstrated in Fig. 1.

When the binding data in Fig. 1 are taken together with the influx/effect data shown in Figs 3 and 4, and Tables 1 and 2, the net result does suggest that 2,4-D pretreatment of artichoke tissue increases the capability of the tissue to retain or bind auxin. This effect itself is curious. If the auxin-binding activity detected here is equivalent to the auxin-binding protein detected in other tissues [1–3], and if this binding protein is indeed the cellular receptor for auxin, then the results published here suggest that in some way treatment with the synthetic auxin, 2,4-D, alters the tissue so that it can now respond to auxin. There is some support for this possibility in the literature. As indicated earlier Libbenga [5] indicated that a soluble auxin receptor may appear after auxin treatment. There is evidence that the maintenance of the auxin transport system (which probably uses auxin-binding proteins) is dependent upon the continued presence of auxin [7]. In its absence the ability of tissues to transport auxin declines. In the case of animal steroids, there is direct evidence that the synthesis of the steroid receptor is induced by steroid treatment of the tissue itself [8]. The generality of such a phenomenon for other growth substances is only speculative. It is notable that the tissue in which auxin receptor proteins are easily detected are those already containing substantial amounts of auxin (e.g. coleoptile) [1–3].

Some final comment must be made on the possible relationship of the tissue changes in auxin-binding to the onset of cell division. In the artichoke tuber tissue used here, division rates were low (Fig. 1b). After 3 days cell numbers had increased only some 70–100% and the first division, occurring between 24 and 36 hr, only occupied some 25–30% of the cells. Clearly an attractive possibility is that the auxin-induced changes in auxin-binding, influx and efflux reflect the differences between non-dividing cells, but this will require substantially more evidence for its proof than that quoted in this paper.

EXPERIMENTAL

Artichoke tissue and culture. Artichoke tubers (cv Bunyards Round) were grown in the Botany Department garden and stored at 4° in sand. After scrubbing off excess soil, tubers were sterilized by dipping for 30 min in a soln of sodium hypochlorite (2% available Cl_2). The tubers were sliced, sterile tissue removed with a 1 cm dia cork borer and the cylinders sliced into 1 mm thick discs. The tissue was incubated by roller culture in darkness at 25° in sucrose/mineral salts at a tissue/medium ratio of 1 g to 5 ml [4]. When included, 2,4-D was used as an auxin at concs between 10^{-5} and 10^{-7} M. Sterility checks were made, both by visual observation of the medium and by placing cultured tissue on 2% sucrose/nutrient broth. Non-sterile experiments were discarded. Cell number determinations were made after incubating the tissue for 2 days in 5% chromic acid.

Specific 'in vitro'-binding auxin by membrane fractions. This was carried out using a mixture of methods from refs. [1] and [2]. Labelled IAA (IAA-[2-^{14}C] sp. act. 54 mCi/mmol, Radiochemical Centre, Amersham) was used for the binding studies. The material supplied was of greater than 98% purity and was stored in toluene-Me_2CO (9:1) under N_2 at −20° in darkness. Under these conditions chemical decay was undetectable in a 3 month experimental period. When needed, samples of IAA-[^{14}C] were dried using N_2 gas at 50°, dissolved in the appropriate buffer, kept cold and dark, and used within 3 hr. Cultured and fresh artichoke tissue was ground in a pestle and mortar in 3 vol. of a soln containing 5% PVP 10 mM ME, 0.1 M Tris pH 7.8, 5 mM $MgCl_2$, 10^{-4} M IAA and 0.1 M KCl. Using the grinding buffer of ref. [2], extensive polyphenol formation occurred and PVP and ME were necessary to prevent this. As explained in the text IAA was included to prevent damage to the binding site by ME. The homogenate was squeezed through 20 μm nylon mesh. The filtrate was centrifuged at 5000 g for 10 min, the pellet discarded and crude membrane fraction sedimented at 100 000 g for 15 min. This pellet was washed twice by re-suspension in 0.5 M Tris (pH 7.4)–1 mM $MgCl_2$, pelleting at the same speed. To measure auxin-binding the membrane fraction was re-suspended to a concn of *ca* 1 mg protein/ml (equivalent to 2.5–3 g fr. wt tissue) in 10 mM sodium citrate buffer (pH 5.5)–10 mM $MgCl_2$. IAA-[^{14}C] was added to a final concn of 2×10^{-7} M. Specific or saturable binding was determined by adding unlabelled IAA to a final concn of 10^{-4} M (with appropriate pH adjustment) to half the extract. The extracts were dispensed in 1 ml quantities into plastic tubes. Determination of specific binding was in each case replicated at least 4 times by sedimenting the membrane fraction at 36 000 g for 45 min. The tubes were allowed to drain for 10 min, the surface of the pellet and sides of the tube washed with 5 ml H_2O and labelled IAA in the pellets extracted with MeOH and counted. Total labelled IAA in the pellet represented some 2–3% of the IAA in the extract, and specifically bound IAA (i.e. that competed out by 10^{-4} M IAA) represented 15–20% of this in membrane fractions from 2,4-D cultured tissue.

Uptake and efflux of labelled 2,4-D. Labelled 2,4-D (2,4-D-[2-^{14}C] sp. act. 30 mCi/mmol) was used for these expts and all incubations were carried out in dim green light at 25°. Tissue, cultured for 0–3 days in the presence and absence of 2,4-D, was washed in sterile H_2O and for uptake measurements was dispensed in batches of 10 discs (*ca* 1 g fr. wt) into sterile Petri dishes. The tissue was labelled by shaking in 5 ml sucrose/mineral salts with 2,4-D-[^{14}C] at 10^{-6} M. Determination of uptake at specific times was made using a 10 disc

batch which was washed briefly in running H_2O and the labelled 2,4-D then extracted with boiling EtOH. Equilibrium was generally reached between 30 and 40 min of the commencement of incubation in all cases when *ca* 12–15% of the labelled 2,4-D was taken up. For efflux expts, batches of 20–30 discs cultured for 0–3 days in the presence or absence of 2,4-D were washed with sterile H_2O and labelled for 0.5 or 2 hr in 15 ml sucrose/mineral salts containing 2,4-D-[^{14}C] at 10^{-6} M. The discs were then briefly washed in sterile H_2O and incubated in 15 ml sucrose/mineral salts containing 10^{-6} M 2,4-D-[^{12}C]. Samples (0.1 ml) of the medium were removed every 10 min for counting and at the end of the incubation residual 2,4-D was extracted with boiling EtOH. Inclusion of 2,4-D in the chase medium had no discernible effects on efflux kinetics but all the results described here have used it.

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