THE EFFECT OF AUXINS ON THE BINDING OF PECTIN METHYLESTERASE TO CELL WALL PREPARATIONS

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Summary

It is shown that the plant auxins 3-indolylacetic acid, 2,4-dichlorophenoxyacetic acid, and α-naphthalene acetic acid are effective in binding pectin methylesterase (PME) to cell wall preparations from tobacco pith and tubers of the Jerusalem artichoke.

Each auxin has an optimum concentration for activity. The activity curves are consistent with the hypothesis that the auxin binds PME to cell wall receptor sites by an adsorption mechanism which can be described by the Langmuir adsorption isotherm.

It is suggested that the auxin-mediated binding of PME could control extension growth by controlling the extent of esterification of the pectic substances of the cell wall.

I. Introduction

In an earlier paper (Glasziou 1957) it was suggested that auxins may exert their effect by helping to bind enzymes of cell wall metabolism to receptor sites within the cell. A technique was described by which a pectin methylesterase (PME)–cell wall complex could be split in salt solution and subsequently recombined in the presence of 3-indolylacetic acid (IAA), with an optimal effect at about 10^{-10}M IAA.

The work presented in this paper extends the observations on the binding of PME to cell wall in the presence of IAA, and includes observations with the growth substances 2,4-dichlorophenoxyacetic acid (2,4-D) and α-naphthalene acetic acid (NAA). The effect of the growth substances is shown to occur in preparations from top internodes of young, actively growing tobacco plants and from the tubers of Jerusalem artichokes, as well as from mature stems of tobacco plants. Some observations on the effects of calcium ions and ethylenediaminetetra-acetic acid (EDTA) are presented, and the possible relationship between the effect of auxins on the binding of PME and their effect on the growth rate of *Avena* coleoptiles is discussed.

II. Methods and Materials

The method described previously (Glasziou 1957) for separating PME from the wall fraction and for reconstituting a wall–PME complex has been followed with minor alterations. In outline the method consisted of isolating a wall–PME complex by differential centrifugation of a tissue homogenate and then splitting the complex with 10 per cent. sodium chloride. The solubilized PME was removed from the wall fraction by filtration and dialysed; the wall fraction was washed extensively to

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remove sodium chloride and residual PME. The two components were recombined by incubating with IAA at one of several concentrations. Uncombined PME and excess IAA were removed by filtration and washing, and the PME activity of the wall fraction was assayed. This technique has been modified by (i) including EDTA at a final concentration of 1×10^{-3}M in the buffer for homogenization of the tissue, (ii) increasing the period used during dialysis of the PME solution to about 18 hr, and (iii) raising the pH for incubation of the wall fraction, PME, and auxin to 7·0.

It has become apparent that the method which yielded consistent results with tissue from mature tobacco stems which had flowered required modification to obtain active preparations from some other tissues. Marked differences in preparations from different plants or tubers have been observed, particularly in the presence of a component in the wall preparation to which PME is adsorbed in the absence of added growth substance. This non-specific adsorption usually decreased with time of storage of wall fraction in the cold, but in one experiment it increased.

Enzyme assay.— The method previously used was modified to permit assay of smaller amounts of PME. The assay mixture consisted of 25 ml 0·5 per cent. citrus pectin (purified by the method of Kertesz 1957), and 1·5 ml 1·0M $CaCl_2$. The pectin solution was prepared freshly each day by being dispersed in a Waring Blendor, filtered, and neutralized immediately prior to use. After addition of the enzyme, the assay system was adjusted to pH 7·5 (glass electrode) with 0·005N NaOH, and then one drop extra of alkali added to raise the pH to about 7·7. A stop-watch was started when the pH returned to 7·5. The pH was continually adjusted to 7·0–7·5 during the course of the assay. The end-point was determined in the same way as the starting point, i.e. the pH was adjusted to 7·7 and the time taken when the pH returned to 7·5. The time interval, which varied between experiments (depending on the activity of the enzyme), was usually 10 min, but occasionally longer. With reasonable care and close attention to the condition of the pH meter and electrodes, the method gives results which are reproducible to within 0·025 ml of the titrating alkali.

III. Results

Figure 1(*a*) shows the effect of IAA on the formation of a wall–PME complex in a preparation from the pith of tobacco plants which had flowered. In Figure 1(*b*) the reciprocal plot of data taken from the curve of Figure 1(*a*) is shown together with the calculated auxin concentration which gives half the maximum adsorption of PME to the cell wall (K_{auxin}). This is similar in derivation to the growth constant of Foster, McRae, and Bonner (1952), which, as they point out, has analogies to a Michaelis constant.

Data for reciprocal plots given in this paper have been obtained from the curves fitted to the experimentally determined points. This procedure was necessary because of large differences in auxin concentrations required to give significant differences in the activity of the wall–PME complex. The justification for such a procedure was that, in seven experiments, a straight line could be drawn as line of best fit in the reciprocal plots and each extrapolated line cut the vertical axis. The shape of the curve proximal to the vertical axis has important theoretical

implications which are discussed later. A value of about 10^{-10}M was consistently obtained (see Fig. 1(*a*)) for the optimal concentration of IAA for the binding of PME to wall preparations from tobacco plants and from artichoke tubers, but in one preparation from tobacco plants, 2–3 ft tall, the optimal concentration was between 10^{-6} and 10^{-7}M. This was the highest value obtained in any experiment.

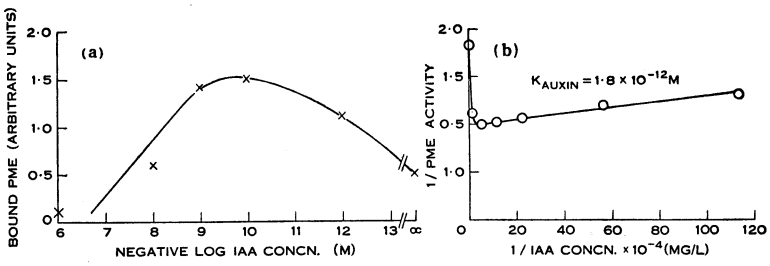

Fig. 1.—(*a*) Effect of IAA on the binding of PME to a cell wall preparation from the pith of tobacco plants which had flowered. (*b*) Reciprocal plot taken from the smooth curve of (*a*) from 5×10^{-9} to 5×10^{-12}M IAA.

Many preparations from tobacco plants 3–5 ft tall and from artichoke tubers showed high non-specific adsorption of the enzyme (adsorption of PME to the wall fraction in the absence of added auxin) which tended to mask the effects of added auxin (Fig. 2). These curves are apparently composite, reflecting both the

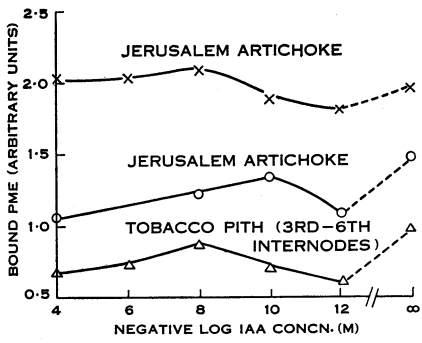

Fig. 2

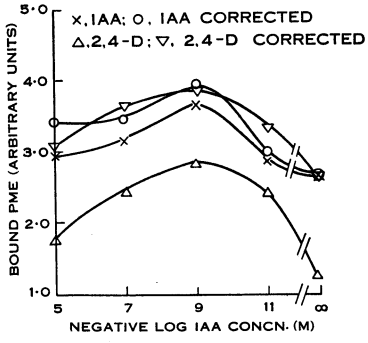

Fig. 3

Fig. 2.—Effect of IAA on the binding of PME to cell wall preparations which show high non-specific adsorption of PME.

Fig. 3.—Effect of IAA and 2,4-D on the binding of PME to a cell wall preparation from the pith of the top internodes of young tobacco plants (see text for explanation of "corrected").

effect of auxin and the non-specific adsorption. It can be seen in Figure 2 that auxin appeared to have a depressing effect on the non-specific adsorption which may be due to the formation of a PME–auxin complex of different adsorption characteristics.

Non-specific adsorption sometimes decreased with time, and when this occurred it was necessary to corect the data to account for changes taking place during the course of the experiment. In Figure 3, the corrected and uncorrected curves are plotted for the effects of IAA and 2,4-D on a preparation from tobacco pith in

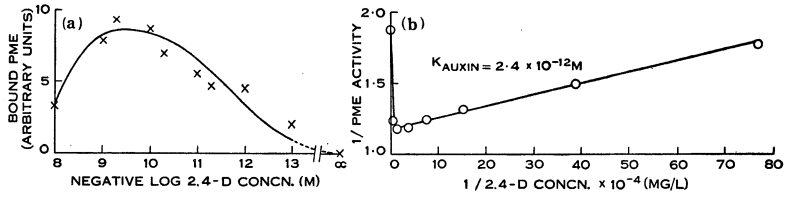

Fig. 4.—(*a*) Effect of 2,4-D on the binding of PME to an EDTA-treated cell wall preparation from tubers of the Jerusalem artichoke. (*b*) Reciprocal plot taken from the smooth curve of (*a*) from 5×10^{-9} to 5×10^{-12}M 2,4-D.

which the non-specific adsoprtion decreased with time. The necessity for these corrections could be avoided if all treatments and assays were carried out simultaneously, when the non-specific adsorption would be more nearly constant in all treatments.

TABLE 1

EFFECT OF 2,4-D AND CALCIUM IONS ON THE BINDING OF PECTIN METHYLESTERASE (PME) TO CELL WALL CONSTITUENTS OF ARTICHOKE TUBERS

The complete system contained 4·0 ml wall suspension, 4·0 ml PME solution, and additions as shown to give a final volume of 10·0 ml. After incubation for 30 min at 22°C the residues were filtered, washed, and the enzyme activity assayed

Experiment No.	2,4-D Concn. (M)	Calcium Ion Concn. (M)	PME Activity (arbitrary units)
1	—	—	0·24
2	1×10^{-7}	—	0·84
3	1×10^{-9}	—	1·00
4	1×10^{-11}	—	1·26
5	—	1×10^{-2}	3·52
6	1×10^{-7}	1×10^{-2}	2·34
7	1×10^{-9}	1×10^{-2}	3·02
8	1×10^{-11}	1×10^{-2}	3·12

The amount of non-specific adsorption varied with the experimental material, and was sometimes negligible. The effect of 2,4-D on a preparation from artichoke tubers which showed very low non-specific adsorption is shown in Figure 4(*a*), and in Figure 4(*b*) the reciprocal plot for 2,4-D concentration between 5×10^{-9} and 5×10^{-12}M is given. The artichokes for this experiment were freshly dug and EDTA was included in the buffer for homogenization of the tissue in the Waring Blendor.

Similar preparations from the same batch of artichokes showed high non-specific adsorption after storing in damp sand in a cold room at 5°C for 1 week. Adamson and Adamson (1957) using the same artichokes found no lag period for expansion in the presence of auxins for tissue from freshly dug immature tubers, but that a lag period developed on storage at low temperature.

At least part of the non-specific adsorption of PME to cell wall preparations may be attributed to the presence of calcium ions. Table 1 gives the effects of 2, 4-D and calcium ions on the reconstitution of the PME–wall complex in the preparation from artichoke tubers, from which it can be seen that the addition of calcium ions at a concentration of 1×10^{-3}M markedly increased the adsorption of PME to the wall fraction. The effect of calcium ions was greatest in the absence of added auxin. Nevertheless, there was a clear auxin effect superimposed on that due to calcium ions. These results compare with those shown in Figure 2 and are presented as further

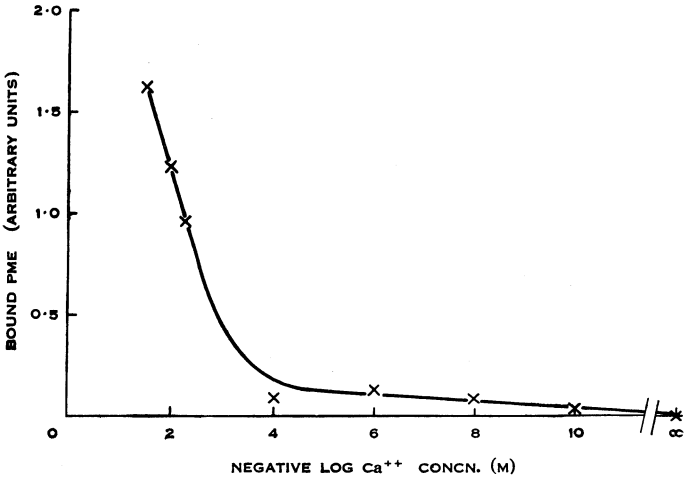

Fig. 5.—Effect of calcium ions on the binding of PME to an EDTA-treated cell wall preparation from tubers of the Jerusalem artichoke.

evidence that auxins alter the adsorption properties of PME, perhaps by the formation of a PME–auxin complex. The effect of varying concentrations of calcium ions on the adsorption of PME to a wall fraction from artichoke tubers is shown in Figure 5. Relatively little effect was observed at concentrations less than 1×10^{-4}M, but a very marked effect was observed at concentrations from about 1×10^{-3} to 3×10^{-2}M Ca^{++}. This effect is not to be confused with the well-known effect of divalent cations on the activation of PME, a possibility guarded against by the extensive washing of the PME–wall complex before assay of the PME activity, and also by the presence of $5{\cdot}7 \times 10^{-2}$M Ca^{++} in the assay system.

That NAA has similar effects to IAA and 2,4-D has been demonstrated on preparations from both young tobacco plants and freshly dug artichoke tubers. In Figures 6(*a*) and 6(*b*) results for an experiment on tobacco pith from the top internodes of plants about 3–4 ft high and the reciprocal plot are given. In experiments with NAA the washing procedure, after reconstitution of the wall–PME com-

plex, was reduced to two washings with 5-ml portions of 0·001M phosphate buffer, pH 7·0. It is not known whether the higher K_{auxin} value for NAA as compared with those obtained for IAA and 2,4-D indicates that NAA has a lower affinity in the system for binding PME to the wall. A decision on these kinetic aspects must await purification of the system components.

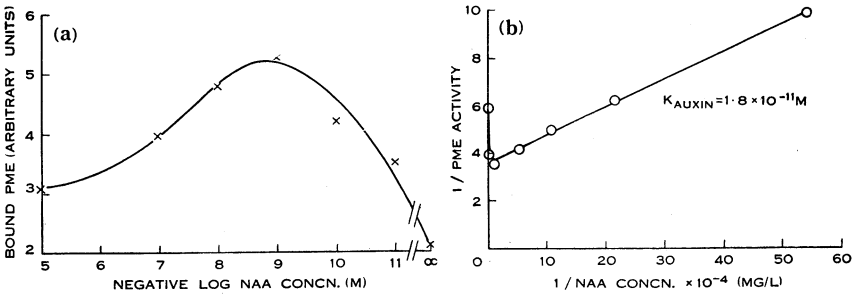

Fig. 6.—(*a*) Effect of NAA on the binding of PME to an EDTA-treated cell wall preparation from the top internodes of young tobacco plants. (*b*) Reciprocal plot taken from the smooth curve of (*a*) from 1×10^{-7} to 1×10^{-11}M NAA.

IV. Discussion

It has been established that the plant growth substances IAA, 2,4-D, and NAA are effective in binding PME to cell wall preparations from mature stems of tobacco plants which had flowered, from top internodes of young rapidly growing tobacco plants, and from tubers of the Jerusalem artichoke. The optimal concentration of growth substance is of the order of 10^{-10}M, and there is a falling-off of the effect at higher and lower concentrations. It can reasonably be presumed that the effect of higher concentrations is an inhibition of the binding of the enzyme rather than a lowering of its activity, as the addition of IAA, 2,4-D, or NAA to the assay system at concentrations above the optimal has no effect on the activity of either the soluble enzyme or the bound enzyme.

The accuracy with which the effect of growth substances on the binding of PME to the cell wall can be measured is not yet sufficiently high to offer conclusive proof that mathematical treatment in terms similar to classical enzyme kinetics would be valid. However, in all cases where sufficient data have been obtained, reciprocal plots of the PME activity of the wall–enzyme complex against the auxin concentrations gave a linear relationship at low concentrations of the auxin and at high concentrations the curve rose sharply. Thus it is a reasonable asusmption, as a basis for discussion, that these results can be treated in a similar manner to that used by Foster *et al.* (1952) for analysing the effect of auxin on coleoptile growth.

Relation to other Work

Heyn (1931) suggested that extension growth under the influence of auxin involves an increase in cell wall plasticity, and Kerr (1951), in considering the properties of cellulose and pectic substances, suggested that the properties of the

primary wall could be explained on the assumption that protopectin forms a continuous phase and the cellulose microfibrils form a discontinuous phase. Bennet-Clark (1955) considered that the pectins of the walls have maximum extensibility when the carboxyl groups are converted to methyl esters, and Ordin, Cleland, and Bonner (1955) have demonstrated that auxin influences the incorporation of methionine-^{14}C, labelled in the methyl group, into the hot-water-soluble non-cellulosic fraction of the cell wall.

Recent electron microscope studies of the microfibrillar pattern of *Avena* coleoptile parenchyma cells (Wardrop 1955, 1956) indicate that during extension growth the wall expands evenly and that new, transversely orientated microfibrils are deposited as growth proceeds. The subsequent disorientation of the microfibrils takes place in a manner consistent with a stretching of the wall, and this may be facilitated by changes in the non-cellulosic cell wall constituents.

The work of Foster *et al.* (1952) and McRae, Foster, and Bonner (1953) has shown that, under certain rigidly defined experimental conditions, the concentration of added growth substance may become rate limiting for the growth of the *Avena* coleoptile. Under such conditions, the growth response was related to the concentration of the growth substance in a manner similar to the relation of rate of enzyme reactions to concentration of substrates for certain enzymes which are inhibited by high concentrations of their substrates. The growth substance was considered to bind at two points to a cellular component to form a growth-active complex. At high auxin concentrations, competition by individual auxin molecules for the attachment sites on the cellular component gave rise to a complex in which two auxin molecules bound, one at each point, were considered to inhibit growth. When the reciprocal of the growth rate was plotted against the reciprocal of the auxin concentration, a straight line was obtained up to the optimal concentration after which the curve rose steeply. The reciprocal plots for the binding of PME to a wall component in the presence of auxins give the same type of curve. It is pertinent to consider whether these two auxin-mediated reactions are actually the same reaction.

It has been proposed previously (Glasziou 1957) that the activity of PME could control the extent of esterification of the pectic substances and so control their elastic and plastic extension properties. To relate the auxin-mediated growth reaction in coleoptiles and the auxin-mediated binding of PME to the cell wall, it is necessary to assume that growth under the conditions used by Bonner's group was inversely related to PME activity, which in turn was related to the concentration of auxin, being progressively decreased by concentrations up to the optimal and increased at supraoptimal concentrations.

Foster *et al.* (1952) pointed out the analogy of their treatment to the Michaelis-Menten hypothesis for enzyme action which assumes the formation of an intermediate enzyme-substrate complex, the rate of the reaction being governed by the number of adsorbed substrate molecules. However, this relationship can also be derived from the Langmuir adsorption isotherm. That the relationship between growth and auxin concentration, and the adsorption of PME to the cell wall can be described by a similar mathematical treatment as that for an enzyme and substrate implies only that all are examples of a type of adsorption phenomenon. The Michaelis

constant, although calculated in the same way, does not have the same significance in the different reaction systems.

Results for the binding of PME in the presence of auxins have been obtained with tobacco pith and the tubers of the Jerusalem artichoke. Adamson and Adamson (unpublished data) have observed that disks from Jerusalem artichoke tubers expand in the presence of auxins (IAA, 2,4-D, and NAA), the rate of expansion (after the lag period) being linear with time over periods of up to 48 hr, and over a range of auxin concentrations above the optimal. Reciprocal plots of the growth rate against auxin concentration give the same kind of relationship as for the *Avena* coleoptile.

A tentative mechanism by which the auxin-mediated growth of plant tissues may be related to the binding of PME are as follows: Firstly, the steady-state extension growth is dependent on the extent of methylation of the pectic susbtances of the cell wall; secondly, the reactions involved in the synthesis and methylation of pectates may be described generally as:

$$\text{Pectates} + \text{"methyl"} \rightarrow \text{pectins} \xrightarrow[\text{PME}]{H_2O} \text{pectates} + \text{methyl alcohol};$$

and, thirdly, the activity of PME is assumed to be controlled by auxin-mediated binding in the following manner:

$$\begin{matrix} \text{Wall} + \text{auxin} \rightarrow \text{wall–auxin} \\ \text{PME} + \text{auxin} \rightarrow \text{PME–auxin} \end{matrix} \begin{matrix} \nearrow + PME \\ \searrow + \text{wall} \end{matrix} \rightarrow \text{wall–auxin–PME}.$$

If the wall–auxin–PME complex is considered to be the ineffective form of PME in the cell, methylation of pectates will be at a maximum when there is maximum binding of PME to the wall sites. At high concentrations of auxin, the decreased concentration of free PME and wall sites would limit the formation of the wall–auxin–PME complex. The enzymatic activity of PME and PME–auxin is the same *in vitro* and may be presumed to be the same *in vivo*. Given these relationships, it is feasible that the rate-limiting reaction for extension growth could be the auxin-mediated binding of PME.

An apparent anomaly in this interpretation is that the optimal concentrations of auxins for coleoptile growth measured by Bonner's group were much higher than the optimal values for the binding of PME to the wall sites. The values for PME-binding were obtained in a simple aqueous system in which the activities (in the physicochemical sense) of the components would not be very different from their actual molar concentrations. However, in the cell the activities of these components are likely to be very different from their molar concentrations due to the complex nature of the cytoplasm, and so the anomaly may not be as serious as it first appears.

The non-specific binding of PME to components of the wall fraction has been partially attributed to the effect of calcium ions, but not all non-specific binding can be explained in this way. Such binding may be either an artifact of no significance in the intact cell or some complementary mechanism controlling PME activity. The effects of calcium ions on binding of PME are of interest because of the relationship between auxins and calcium ions on the extension growth of *Avena* coleoptiles

(Bennet-Clark 1955) and the effects of chelating agents (Heath and Clark 1956). Also Burström (1954) has observed that the effect of calcium ions in root growth resembles that of an anti-auxin, increasing the cell elongation and apparently antagonizing an auxin. Some aspects of these effects may be explained if the wall–Ca^{++}–PME complex is less effective in reducing the activity of PME than is the wall–auxin–PME complex. These relationships are complicated by the effect of calcium ions on the physical properties of pectates and the fact that calcium ions are powerful activators of PME.

The proposed mechanism by which the effect of auxin on the binding of PME could become manifested in cell elongation is only one of several which could be advanced. Further evidence is required on the effects of other auxin-like compounds (particularly competitive inhibitors of auxin action) on the adsorption of PME to the wall sites. However, it will be necessary to purify the components of the system to obtain conclusive results in experiments of this type. Work along these lines is now in progress and will be reported in a subsequent paper.

V. Acknowledgments

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