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EFFECTS OF 2,4- AND 3,5-DICHLOROPHENOXYACETIC ACIDS ON JERUSALEM ARTICHOKE TUBER TISSUE DISKS

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Abstract—Treatment of Jerusalem artichoke tuber tissue disks with 10^{-5} M solutions of the auxin 2,4-dichlorophenoxyacetic acid (2,4D) induced marked physiological and biochemical responses. There was a large uptake of water, considerable hydrolysis of oligosaccharides, and a very significant increase in hydrolase and invertase activity. Similar treatment with the isomer 3,5-dichlorophenoxyacetic acid (3,5D), which is inactive as an auxin, did not produce these responses. Possible explanations for the activity of 2,4D are discussed.

INTRODUCTION

THE PHYSIOLOGICAL responses to plant growth regulating substances have been intensively studied over many years using several types of plant tissue. Attempts to relate the physiological responses to changes in the biochemistry of the tissue, and in particular to changes in the activity of certain enzymes, have met with little success because the response was usually very small or limited to the peripheral cells of the tissue. However, in 1960 Yomo[1] and Paleg[2] found independently that the induction of α-amylase in the embryo-free grain of barley was stimulated by gibberellin. This was subsequently confirmed by several workers,[3,4] but there was still no clear evidence for the effect of an auxin on an enzyme system.

The demonstration that growth substance treatment of tissue disks cut from certain inulin-storing roots and tubers, such as Jerusalem artichoke, chicory and dandelion, induced a very large uptake of water,[5] provided here a source of suitable material in sufficient amounts for enzymic study. In studies with Jerusalem artichoke it was found that the water uptake induced by treatment with 10^{-5} M solutions of the highly active plant growth regulator 2,4-dichlorophenoxyacetic acid (2,4D) was associated with the hydrolysis of oligosaccharides to simpler sugars.[6] This system was therefore used for studies on the effects of growth regulator on enzymes of carbohydrate metabolism. Preliminary work[8] indicated that enzyme preparations from 2,4D-treated Jerusalem artichoke tissue disks showed a marked increase in invertase and hydrolase activity and these enzymes were therefore chosen for detailed study. (See also Edelman and Jefford[7].)

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RESULTS

Figure 1 shows the effect of duration of treatment on the magnitude of water uptake induced in disks cut from Jerusalem artichoke tubers when treated with either water or 10^{-5} M solutions of the inactive growth regulator 3,5-dichlorophenoxyacetic acid (3,5D) and 2,4D. The water and 3,5D treatments induced only a small uptake of water and this occurred mainly during the first 24 hr; 10^{-5} M solutions of 2,4D, on the other hand, caused large amounts of water to be taken up for at least 4 days.

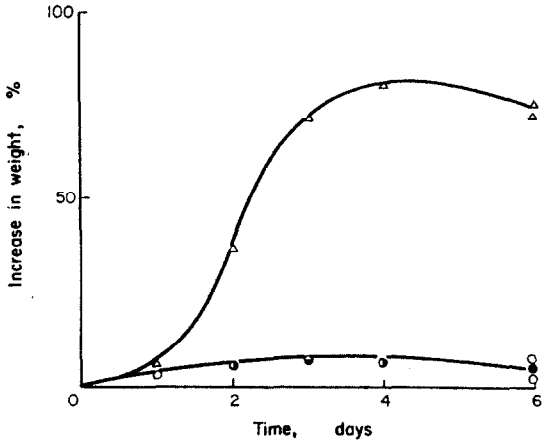

FIG. 1. THE WATER UPTAKE INDUCED IN JERUSALEM ARTICHOKE TISSUE DISKS AT 25° BY TREATMENT WITH WATER OR 10^{-5} M SOLUTIONS OF EITHER 2,4D OR 3,5D FOR VARYING PERIODS OF TIME.
△ 10^{-5} M 2,4D. ● 10^{-5} M 3,5D. ○ Water.

TABLE 1. INSOLUBLE SUGARS AS A PERCENTAGE OF DRY MATTER; DIRECT DETERMINATION FOR EXTRACTS FROM JERUSALEM ARTICHOKE TISSUE DISKS TREATED WITH 10^{-5} M 2,4D, 10^{-5} M 3,5D AND WATER AT 25°

Duration of treatment (days)	Treatment		
	10^{-5} M 2,4-D	10^{-5} M 3,5-D	Water
0	6·0	6·0	6·0
1	3·5	—	2·8
2	3·4	2·7	3·2
3	4·2	4·3	4·5
4	3·5	4·0	5·1
6	3·1	4·9	5·2

Table 1 and Figs. 2 and 3 show the changes in reducing, total soluble and insoluble sugars after various times of treatment with water, 10^{-5} M 3,5D and 10^{-5} M 2,4D. None of the treatments produced a significant change in the insoluble sugar content of the disks (Table 1). Both water and 3,5D treatment caused a small decrease in total soluble sugars and this was accompanied by a slight increase in reducing sugar content. When 10^{-5} M solutions of 2,4D were used there was a large decrease in the percentage of total soluble sugars but the reducing

sugar content of this sugar mixture increased considerably, especially during the second and third day of treatment.

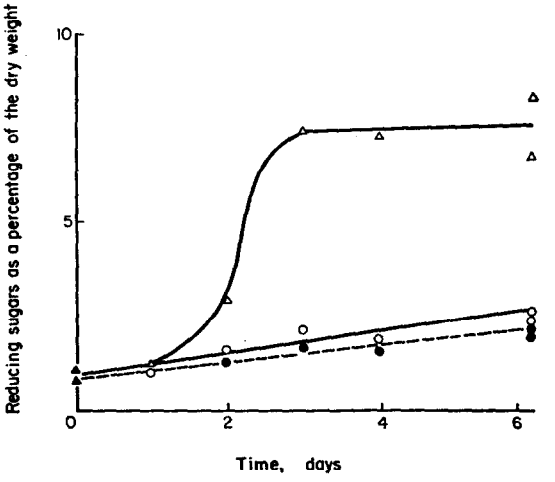

FIG. 2. THE CHANGES IN PERCENTAGE OF REDUCING SUGARS IN JERUSALEM ARTICHOKE TISSUE DISKS AT 25° AFTER TREATMENT WITH WATER OR 10^{-5} M SOLUTIONS OF EITHER 2,4D OR 3,5D FOR VARYING PERIODS OF TIME.

△ 10^{-5} M 2,4D. ● 10^{-5} M 3,5D. ○ Water.

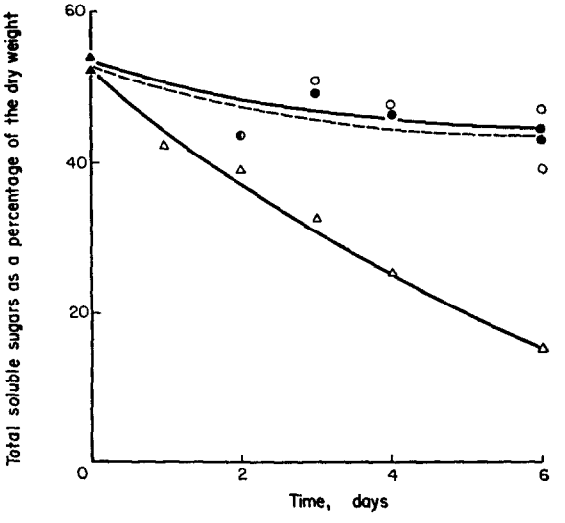

FIG. 3. THE CHANGES IN PERCENTAGE OF TOTAL SOLUBLE SUGARS IN JERUSALEM ARTICHOKE TISSUE DISKS AT 25° AFTER TREATMENT WITH WATER OF 10^{-5} M SOLUTIONS OF EITHER 2,4D OR 3,5D FOR VARYING PERIODS OF TIME.

△ 10^{-5} M 2,4D. ● 10^{-5} M 3,5D. ○ Water.

Table 2 and Fig. 4 illustrate the results obtained from chromatographic analysis of the total soluble sugar fraction. Separation of sugars whose molecular weight exceeded that of a pentasaccharide was not always satisfactory so the results for all the higher sugars were combined and are presented in Table 2. Figure 4a shows the variation in fructose content with

TABLE 2. SOLUBLE SUGARS (ABOVE PENTASACCHARIDE) AS A PERCENTAGE OF DRY MATTER FROM CHROMATOGRAMS FOR EXTRACTS OF JERUSALEM ARTICHOKE TISSUE DISKS TREATED WITH 10^{-5} M 2,4D, 10^{-5}M 3,5D AND WATER, AT 25°

Duration of treatment (days)	Treatment		
	10^{-5} M 2,4D	10^{-5} M 3,5D	Water
0	9·4	9·4	9·4
1	5·7	—	4·2
2	4·1	5·3	4·1
3	4·9	7·1	—
4	1·5	7·0	7·1
6	3·1	6·5	6·9

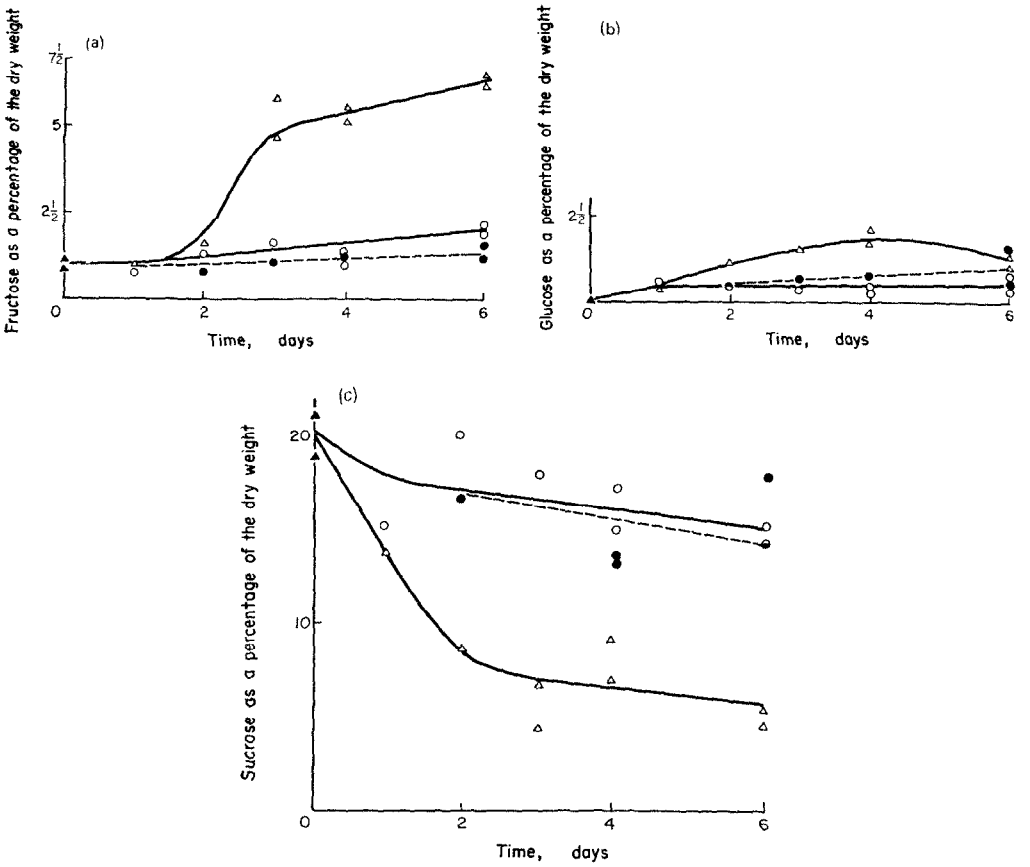

FIG. 4. THE CHANGES IN PERCENTAGE OF SOME OF THE INDIVIDUAL SOLUBLE SUGARS PRESENT IN JERUSALEM ARTICHOKE TISSUE DISKS AT 25° AFTER TREATMENT WITH WATER OR 10^{-5} M SOLUTIONS OF EITHER 2,4D OR 3,5D FOR VARYING PERIODS OF TIME.

(a) Fructose. (b) Glucose. (c) Sucrose.

△ 10^{-5} M 2,4D. ● 10^{-5} M 3,5D. ○ Water.

time and this follows a similar pattern to the corresponding total reducing sugars. In both instances, water and 3,5D treatments produced little change, whereas treatment with 10^{-5} M solutions of 2,4D caused a considerable increase in the amount of fructose present in the disks. Glucose comprised only about a quarter of the total reducing sugar present in the disks after treatment (Fig. 4b) and because the amounts were small it was difficult to determine whether any of the treatments produced significant differences in the glucose content. All the remaining soluble sugars followed a similar decrease in amount with time. Treatment with either water or 10^{-5} M solutions of 3,5D led to a small reduction in amounts of sugar throughout 6 days of treatment, whereas treatment with 10^{-5} M solutions of 2,4D caused a much greater reduction in amounts of sugar particularly during the first 2–3 days of treatment (Fig. 4c and Table 2).

Since treatment of tissue disks with 3,5D gave a similar pattern of water uptake and sugar change to disks treated with water only, a single experiment was carried out to examine the

TABLE 3. ENZYME ACTIVITY AND AMOUNT OF PROTEIN PRESENT IN EXTRACTS FROM JERUSALEM ARTICHOKE DISKS AT 25°

Treatment	Time (days)	Water uptake (%)	Mg protein $\times 10^3$ per mg dry tissue	Invertase activity: Per mg initial dry weight	Invertase activity: Per g final protein	Hydrolase activity: Per mg initial dry weight	Hydrolase activity: Per g final protein
Untreated	—	—	13·9	0	0	436	31·4
Water	3	24	10·2	0	0	351	34·4
10^{-5} M 2,4D	3	96	15·2	324	21·3	1011	66·5

1 unit of invertase activity represents the liberation at 25° of 2 μmoles of hexose/min.
1 unit of hydrolase activity represents the liberation at 25° of 1 μmole of hexose/min.

changes in amount of protein, invertase and hydrolase activity resulting from treatment with a 10^{-5} M solution of 3,5D. The results showed that neither the amount of protein nor the enzyme activity of the disks when treated with 3,5D were greatly different from water-treated tissue. The protein, hydrolase and invertase levels in extracts obtained from the tissue disks after 3 days of treatment with either water or 10^{-5} M solutions of 2,4D are shown Table 3 where the results obtained from untreated disks are also given. Each value is the average of results from five to ten separate experiments. Table 3 shows that water treatment has no significant effect on hydrolase activity whilst 2,4D produces a 2–3-fold increase. Considerable invertase activity was detected after treatment of the disks with 10^{-5} M solutions of 2,4D, whereas none could be detected either in untreated or water-treated extracts.

Fractionation of the protein extracts gave results which followed a very similar pattern to those described by Edelman and Jefford.[7] Hydrolase activity was resolved into two fractions A and B. There was some overlap of invertase protein with hydrolase B, but no attempt was made to achieve further separation. Table 4 shows the invertase and hydrolase activities associated with the two peaks. Nearly all the increase in hydrolase activity after 2,4D treatment is associated with hydrolase B.

TABLE 4. ACTIVITIES OF PROTEIN FRACTIONS OBTAINED FROM EXTRACTS OF JERUSALEM ARTICHOKE DISKS AT 25°

Treatment	Activity in hydrolase peaks* (per mg initial dry wt.)			
	A	B	Total A and B	Average unfractionated
Invertase				
Untreated	0	0	0	0
Water, 3 days	0	0	0	0
10^{-5} M 2,4D, 3 days	2·3	335·1	337·4	324·2
Hydrolase				
Untreated	373·6	142·7	516·3	435·9
Water, 3 days	202·6	178·1	380·7	350·8
10^{-5} M 2,4D, 3 days	298·1	696·8	994·9	1010·5

* See Table 3.

DISCUSSION

Treatment of Jerusalem artichoke tissue disks after storage at 3° for between 6 and 7 months, with either 10^{-5} M solutions of the inactive growth regulator 3,5D or with water has comparatively little effect on either the water uptake or the sugar present in the tissue. However, treatment of the disks with 10^{-5} M solutions of the highly active growth regulator 2,4D not only induces a large uptake of water but leads to a considerable decrease in the percentage of total soluble sugar. This effect, and that described below on enzyme activity, is therefore an auxin effect. The decrease in the molar concentration of total soluble sugars due to 2,4D treatment is minimized by the very large increase in the molar amount of reducing sugars, mainly fructose, that occurs after 2 days. Figure 5 shows that the molar concentration of total soluble sugars falls by over 60 per cent during the first 2 days of treatment, but thereafter the decrease is very small.

The changes in carbohydrate composition that arise as a result of 2,4D treatment, strongly indicate possible changes in enzyme activity associated with carbohydrate metabolism. Since the main substrates remaining after cold storage are sucrose and oligosaccharides consisting of extra fructose units attached to the sucrose, the enzymes initially studied were invertase and hydrolase, both of which have been shown to be active by Edelman and Jefford.[7] The striking increases in activity obtained for the protein extracts from 2,4D-treated Jerusalem artichoke tissue disks lie well outside the values obtained for either water or 3,5D-treated disks (maximum standard deviations $\pm$ 10 per cent). However, it is difficult to state with any certainty whether the small differences in the amounts of protein (maximum standard deviation $\pm$ 20 per cent) observed after the various treatments are real or due to experimental variation. It is therefore impossible to determine whether the large increases in enzyme activity caused by 2,4D treatment are due to the production of new protein, but if this were so its specific activity would have to be very high to account for the observed increase in activity produced by treatment with 2,4D solutions.

The very marked increase in enzyme activity could be explained if the 2,4D treatment loosens enzyme protein, especially that possessing invertase and hydrolase B activity, from

the cell wall. Edelman and Hall[9] have shown that invertase activity of over 1000 units develops in aged tissue disks of Jerusalem artichoke but this resists all attempts at solubilization and appears to be firmly bound to the cell wall. The enhancement of enzyme activity found in extracts after treatment with solutions of 2,4D could be due to the loosening of a considerable amount of the invertase associated with the cell wall and it is this which appears in the extracts after 2,4D treatment as soluble invertase with an activity of 324 units (Table 3). It is not unreasonable to suppose that a similar behaviour could occur with hydrolase B in disks treated with 2,4D solutions.

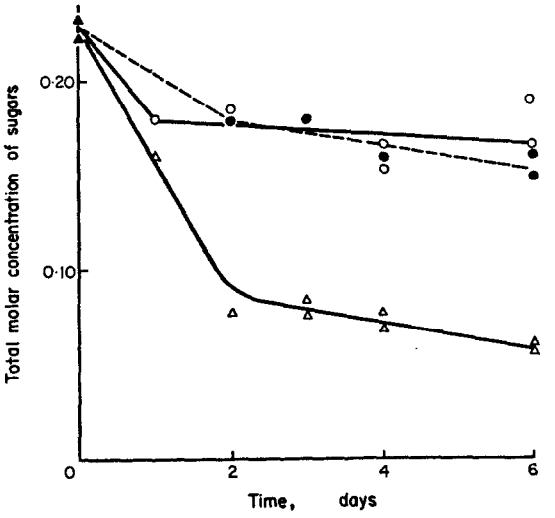

FIG. 5. THE EFFECT OF TREATMENT WITH WATER OR 10^{-5} M SOLUTIONS OF EITHER 2,4D OR 3,5D ON THE MOLAR CONCENTRATION OF SUGARS PRESENT IN TISSUE DISKS OF JERUSALEM ARTICHOKE AT 25°.

△ 10^{-5} M 2,4D. ● 10^{-5} M 3,5D. ○ Water.

EXPERIMENTAL

Biological Material and Treatment

Jerusalem artichoke tubers were obtained from F. A. Secrett, Ltd., Milford, Surrey, and stored in moist peat in a cold store for 6–7 months at 3 ± 1°. The preparation of tissue disks and treatment of the latter with chemicals followed methods already described by Rutherford *et al.*[5]

Extraction and Chromatographic Examination of Sugars

The methods used were those described by Rutherford and Weston.[10]

Determination of Water Uptake and Sugars

Determinations of the water uptake and amount of sugars present were made after 0, 1, 2, 3, 4 and 6 days treatment. For each determination six different tubers were used. From each tuber, forty-two disks were cut and weighed individually (*ca.* 0·5 g each). Seven disks were put into each of six Petri dishes, two containing water, two a solution of 2,4D (10^{-5} M) and two a solution of 3,5D (10^{-5} M). Of the fourteen disks that had come from the same tuber and treated with the same solution, six disks were selected with water uptake closest to the average. Their soluble reducing, total soluble and insoluble sugar contents were determined and then the individual sugars were determined chromatographically.

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Protein Preparation from Tissue Disks

These were obtained by a method based on that described by Edelman and Jefford.[7] Disks (30–40 g initial fresh weight) were extracted in a juice extractor (Moulinex) that incorporated an automatic grater and a basket-type centrifuge. Extraction was made in the presence of 100 mM phosphate buffer, pH 7·6, containing 100 mM cysteine and 100 mM sodium diethyldithiocarbamate. This and all subsequent operations were conducted at 2°. After extraction, the residue was removed from the centrifuge, ground in a mortar and transferred to a small filter press. A little of the phosphate buffer was added and the fluid squeezed out in the press. This operation was repeated twice more. In all, 25 ml of phosphate buffer were used for the whole extraction process. The total extract was dialysed overnight against 20 mM phosphate buffer, pH 7·6, containing 5 mM cysteine and 5 mM sodium diethyldithiocarbamate (six changes) and the non-diffusible material centrifuged at 25,000 × *g* for 20 min to remove an insoluble residue. Protein was precipitated from the supernatant by the careful addition of $(NH_4)_2SO_4$ to 95% saturation, the pH being maintained at 7·6. After standing at 2° overnight, the precipitated protein was separated by centrifugation at 15,000 × *g* for 30 min and then dissolved in 5 mM phosphate buffer, pH 7·6. This solution was dialysed overnight against 5 mM phosphate buffer, pH 7·6 (six changes). The non-diffusible material was centrifuged at 25,000 × *g* for 20 min and the clear supernatant used for analysis.

Chromatography of Protein

A 1 g column, 100 × 10 mm (dia.) of DEAE cellulose (Whatman Chromedia DE11 powder form) was prepared as described by Edelman and Jefford.[7] An aliquot of the protein solution containing *ca.* 20 mg protein was placed on the column and fractionated by elution with the following sequence of solutions of increasing ionic strength: 5 mM, 20 mM, 50 mM phosphate buffer followed by solutions of 50 mM phosphate buffer containing 0·1 M, 0·2 M and 0·3 M NaCl. All these eluents were adjusted to pH 7·6 except the last which was adjusted to pH 8·0. 20 ml of each eluent were used and 10-ml fractions of the column effluent were collected automatically. Each fraction was assayed for invertase and hydrolase activity.

Enzyme Assays

Invertase activity was measured by setting up digests of 4 ml enzyme solution, 1 ml 0·5 M acetate buffer, pH 5·0, containing 0·1 M sucrose, incubating for 4 hr at 25° and measuring the amount of reducing sugar formed by the method of Somogyi.[11] Sugar determinations were done in duplicate. Hydrolase activity was measured in precisely the same manner except that sucrose was replaced with substrate M described by Edelman and Jefford[7] and containing the equivalent of 224 mg fructose in each enzyme digest.

Enzyme Units

Units of hydrolase activity are expressed as μmoles of hexose liberated/min at 25° and units of invertase activity as the number of 2 μmoles of hexose liberated under the same conditions.

Protein Determination

Measurement of soluble protein in the unfractionated extracts was based on micro-Kjeldahl determination of nitrogen, whereas the amount in fractions from the column was determined by the method of Warburg and Christian.[12]

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