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2,4-DICHLOROPHENOXYACETIC ACID AND THE *DE NOVO* SYNTHESIS OF INVERTASE IN CHICORY ROOT TISSUE

ANTHONY J. GORDON* and ALAN E. FLOOD

Department of Physical Sciences, Wye College (University of London), Nr Ashford, Kent, U.K.

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Key Word Index—*Cichorium intybus*; Compositae; chicory root; invertases; 2,4-D; protein synthesis.

Abstract—Using a dual radioactive labelling technique, the large 2,4-D induced increase in invertase activity in root tissue of chicory (*Cichorium intybus*) could not be attributed to *de novo* protein synthesis. The highly active enzyme could have arisen by modification of an inactive enzyme precursor.

INTRODUCTION

When chicory root tissue is treated with 10^{-5} M solutions of 2,4-D, considerable uptake of water occurs [1] which is associated with hydrolysis of inulin to low MW fructosans and reducing sugars, and also with a large increase in invertase activity [2].

Two forms of invertase have been observed in 2,4-D treated tissue which can be separated by DEAE-cellulose chromatography [3, 4]. In contrast, only one invertase can be found in chicory root tissue treated with water, or with the inactive analogue, 3,5-dichlorophenoxyacetic acid (3,5-D) [3]. The additional, highly active enzyme occurs as a direct result of 2,4-D treatment. The origin of this enzyme may therefore help in an understanding of the mode of action of auxins in general. Inhibitors of protein synthesis had reduced the effectiveness of 2,4-D in promoting water uptake and increasing invertase activity, suggesting that the highly active invertase might arise by *de novo* synthesis of enzyme protein [5]. We report here experiments designed to assess the significance of *de novo* synthesis of invertase protein due to 2,4-D treatment of chicory root tissue. A dual radioactive labelling techique was used similar to that described by Patterson and Trewavas [6] and Arias *et al.* [7].

RESULTS AND DISCUSSION

The separation of crude protein by chromatography on DEAE cellulose is shown in Fig. 1. The pattern of protein peaks and the position of invertase from chicory root tissue treated with water or 2,4-D was quite reproducible. Incorporation of leucine-[^{3}H] into the protein comprising these peaks is also shown in Fig. 1.

It will be noted that (a) the invertase activity extracted from water-treated tissue was eluted almost exclusively in peak 3, (b) most invertase activity extracted from tissue treated with 2,4-D was eluted in peak 2A (the amount of activity in peak 3 being little changed by the action of 2,4-D), (c) most radioactivity was recovered in protein from peak 3 with lesser amounts in peaks 1, 2A and 2B.

The assessment of *de novo* protein synthesis was made by measuring the ratio of incorporation of leucine-[^{3}H] to leucine-[^{14}C]. The rationale behind this method is as follows. During the incubation of chicory root tissue with 2,4-D in the presence of leucine-[^{3}H], ^{3}H will become incorporated into any protein which is synthesized. Similarly if tissue disks are incubated with H_2O containing leucine-[^{14}C], ^{14}C will become incorporated into any protein synthesized during this treatment. If proteins are synthesized due to 2,4-D, which are not synthesized due to the presence of water alone, then these proteins will contain ^{3}H but no, or very little ^{14}C. Thus by measuring the relative amounts of ^{3}H and ^{14}C one can assess the effect of 2,4-D on protein synthesis. 'TC' represents the protein extracted from root tissue treated with 2,4-D plus leucine-[^{3}H] (T) combined with an equal weight of tissue treated with H_2O plus leucine-[^{14}C] (C). The control (CC) was derived from the combination of one set of tissue treated with H_2O containing leucine-[^{3}H] with another set treated with H_2O containing leucine-[^{14}C]. The reciprocal treatment of T with leucine-[^{14}C] and C with leucine-[^{3}H] was not carried out.

The amount of ^{3}H and ^{14}C was determined for each fraction eluted from DEAE-cellulose columns and the ratio of ^{3}H:^{14}C for CC and TC, protein calculated. Whereas a constant ratio was obtained for CC, protein (as would be expected from the combination of two sets of tissue treated in the same manner), the effect of 2,4-D was to increase the amount of ^{3}H incorporated compared with the leucine-[^{14}C] control tissue and also to change the pattern of protein synthesis. However the area containing invertase activity due to 2,4-D treatment (peak 2A) did not contain significantly more leucine-[^{3}H] than leucine-[^{14}C].

The fractions in peak 2A which contained the invertase activity (due to the action of 2,4-D) were collected and the proteins separated by electrophoresis on polyacrylamide gel slabs. The position of invertase

*Present address: Department of Plant and Crop Physiology, The Grassland Research Institue, Hurley, Nr Maidenhead, Berkshire, SL6 5LR, U.K.

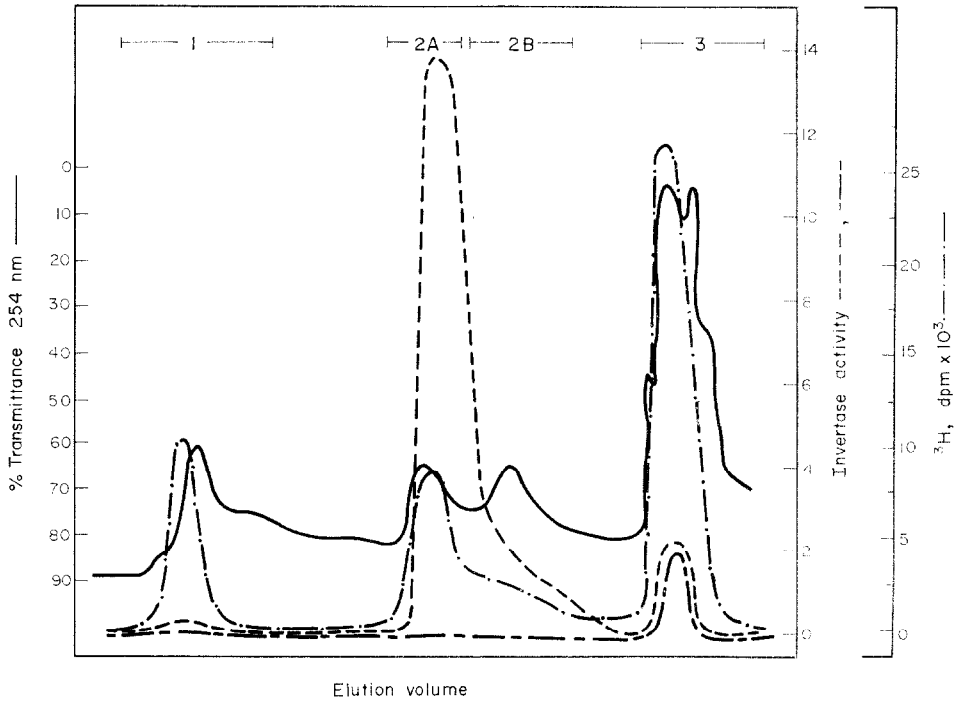

Fig. 1. Separation of crude protein on Whatman DE 52 cellulose column. Peaks of protein were eluted in stages: peak 1 with 5 mM buffer pH, 7.6; peaks 2A and 2B with 50 mM phosphate buffer, pH 7.6; peak 3 with 50 mM phosphate buffer, pH 7.6 containing 0.5 M NaCl. Radioactivity due to leucine-[^{3}H] (·—·); invertase activity (nkat) (– – –) (2,4-D treated tissue); (– – – –) water-treated tissue; and protein (——).

was established on every gel and the ^{3}H:^{14}C content of the protein determined. Fig. 2 shows the typical protein band pattern which became evident upon staining with Coomassie brilliant blue. The protein in areas 'a', 'b', 'c' and 'd' was eluted and the invertase activity, ^{3}H and ^{14}C content and ^{3}H:^{14}C ratio for each area are shown in Table 1. Most of the invertase activity was situated between bands 'a' and 'c' (area 'b' in Fig. 2) but this protein did not contain significantly more ^{3}H than ^{14}C.

In peak 2 A (DEAE-cellulose column) the invertase activity due to 2,4-D was 60 times greater than that due to water treatment of chicory root tissue [4]. If 2,4-D (in the presence of leucine-[^{3}H]) caused the synthesis of the protein responsible for this activity, one might expect that the ratio of ^{3}H:^{14}C would be at least as great as the ratio of the invertase activities due to the two treatments. The inability to find a high ^{3}H:^{14}C ratio associated with the invertase protein eluted from polyacrylamide gel slabs strongly suggested that the invertase was not synthesized *de novo*. However, there was still a possibility that the radioactivity in area 'b' associated with the invertase might be masked by that of other contaminating proteins. The protein from this area was further separated

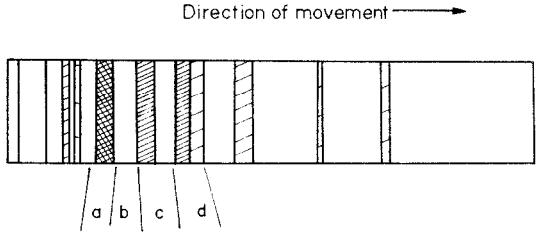

Fig. 2. Protein band pattern obtained by polyacrylamide gel electrophoresis of the protein obtained from peak 2A from a Whatman DE 52 cellulose column.

Table 1. Radioactivity in protein eluted from polyacrylamide gels following electrophoresis of TC peak 2A protein

Experiment No.	Band	% Total inverse recovered	Radioactivity (dpm) ^{3}H	^{14}C	^{3}H:^{14}C ratio
1	(a)	8.4	1270	720	1.75
	(b)	57.6	1280	950	1.34
	(c)	27.9	3210	1220	2.64
	(d)	6.1	1390	1300	1.07
2	(a)	27.5	1320	1220	1.08
	(b)	52.1	1360	750	1.81
	(c)	15.9	1370	870	1.58
	(d)	4.5	1240	750	1.66
3	(a)	40.1	1490	1450	1.02
	(b)	46.6	1280	880	1.46
	(c)	9.7	1660	1190	1.39
	(d)	3.6	1740	1310	1.33
4	(a)	41.9	9630	11100	0.87
	(b)	44.4	5190	4470	1.16
	(c)	11.0	7180	5420	1.33
	(d)	2.7	7570	6720	1.13

Details of the electrophoresis technique and the detection of invertase activity are described in refs. [4] and [22].

Table 2. Radioactivity in protein eluted from duplicate SDS polyacrylamide gels

Gel: Band	dpm ^3H	^{14}C	$^3H/^{14}C$
1 A	325	406	0.80
B	332	344	0.97
C	159	222	0.72
2 A	244	227	1.07
B	154	182	0.85
C	149	173	0.86

on sodium dodecyl sulphate (SDS) polyacrylamide gels [4]. Three bands were evident when stained with Coomassie brilliant blue, and by reference to a standard curve of protein MW against mobility, the MWs were found to be *ca* 46 000, 59 000 and 71 000. The protein of MW 59 000 (band B in Table 2) was most probably the invertase (cf. ref. [4]).

Elution of the protein bands [8] from unstained replicate gels, followed by determination of the amounts of ^3H and ^{14}C present, produced the results shown in Table 2. Again there was no high $^3H:^{14}C$ ratio. From these results it seems unlikely that the increase in invertase activity due to 2,4-D treatment of chicory root tissue can be accounted for by *de novo* synthesis of invertase protein.

The activities of some plant enzymes alter on treatment of the tissues with growth substances [9–13], but there is little evidence to suggest a direct allosteric effect. Using inhibitors, it has been demonstrated that the effects of growth substances depend on RNA and protein synthesis. In only few cases, however, has it been demonstrated that increased enzyme activity due to growth substance treatment came about by *de novo* synthesis of enzyme protein [13].

The present study was initiated to investigate the possibility that the increased invertase activity due to 2,4-D treatment of chicory root tissue, occurred by *de novo* synthesis of enzyme protein. Using the dual labelling technique described, in conjunction with techniques to purify the invertase protein, we were unable to show that invertase protein synthesis occurred to a greater extent in the presence of 2,4-D than with water alone. Confirmation of this result might be sought using density labelling centrifugation.

If there is no *de novo* synthesis of invertase in 2,4-D treated chicory root, an alternative explantion of the increased activity may be that 2,4-D causes the modification of a preformed inactive invertase precursor. The basis for such a speculation has already been put forward [4] and related to this idea is the possibility that an invertase inhibitor may be involved [14–21].

EXPERIMENTAL

Chicory roots (*Cichorium intybus* L.) cv Magdeburg were obtained fron F. G. Harrison, Bury St. Edmunds, Suffolk in November. Undamaged roots were stored at 3±1° in moist peat for at least 4 weeks before use. The preparation of tissue disks from the roots and their incubation with H_2O or 10^{-5} M solns of 2,4-D has been described [20]. In order to reduce the possibility of infection the stainless steel knives and cork borers used in the preparation of tissue disks were flame-sterilized. All glassware and filter papers were heat-sterilized and solns prepared from freshly boiled glass dist. H_2O. Plant material was not treated with any sterilizing agent [20]. The extraction and purification of invertase protein has been described in detail elswhere [4, 21, 22].

Dual radioactive labelling. Equal initial wts of tissue disks were incubated with either H_2O of 10^{-5} M solns of 2,4-D. After 24 hr the disks were transferred for a further 24 hr period to similar solns containing leucine-[4,5-^3H] (5 μCi per ml; 19 Ci/mmol; 2,4-D treated tissue, 'T') or leucine-[U-^{14}C] (5 μCi per ml; 340 mCi/mmol; H_2O-treated tissue, 'C'). At 48 hr the tissue from the two treatments was combined and the protein extracted. This protein is designated 'TC'. A control prepn was obtained by extraction of protein from two further sets of tissue disks of equal initial wt. One set of disks was treated with H_2O containing leucine-[4,5-^3H] (5 μCi per ml; 19 Ci/mmol), and the other set with H_2O containing leucine-[U-^{14}C] (5 μCi per ml; 340 mCi/mmol). This protein is designated 'CC'.

Analysis of radioactivity. (a) *In fractions eluted from DEAE-cellulose columns* (*Whatman DE* 52). Fractions were dialysed against 5 mM triethylamine carbonate buffer, pH 7.6 [6] and then transferred to individually marked scintillation vials and freeze-dried. Residue was solubilized in H_2O (50 μl) and Nuclear Chicago Solubilizer (0.5 ml). The vials, tightly capped, were incubated at 50° for 4 hr and then for 18 hr at room temp. before scintillation liquid (10 ml, 5 g PPO/l. toluene) was added. (b) *In protein on polyacrylamide gels.* Protein was detected and eluted from polyacrylamide gels as described in refs. [4] and [22]. After freeze-drying, samples were solubilized and scintillation liquid added as above. (c) *In protein on SDS polyacrylamide gels.* Protein was eluted as described in ref. [8] and then prepared for counting as outlined above.

Scintillation counting. Each sample was counted in the ^3H and the ^{14}C channels of a Beckman LS 100 scintillation counter. Corrections for quenching were made by reference to quench curves for each channel using the ext. standard ratio method. The $^3H:^{14}C$ ratios were calculated from such corrected counts.

Invertase activity. Assay is described in ref. [4].

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