Phytochemistry, Vol 22, No 1, pp 37–45, 1983
Printed in Great Britain

0031–9422/83/010037–09$03 00/0

PHOTOINDUCED CHANGES IN *o*-DIPHENOL OXIDASE AND *p*-COUMARATE HYDROXYLASE ACTIVITIES IN SPINACH BEET SEEDLINGS AND LEAVES

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(*Received* 29 *March* 1982)

Key Word Index—*Beta vulgaris*, Chenopodiaceae, spinach beet, phenylpropanoid metabolism, photoinduction, phenolase, *p*-coumarate 3-hydroxylase

Abstract—Whereas illumination of mature leaf discs and prolonged illumination of spinach beet seedlings gave rise to co-ordinated increases in PAL and C4H followed later by increases together in *p*C3H and *o*-diphenol oxidase, relatively brief illumination of seedlings with white or red light gave co-ordinated increases in PAL, C4H and *p*C3H without any increase in DOPA oxidase. The *p*C3H induced in young seedlings has been partially purified and shown to be a copper protein with relatively low DOPA oxidase activity, but chromatographically separable from other phenolases present. The enzyme was similar to these other phenolases but distinguished by its inability to catalyse the 3-hydroxylation of tyrosine. This phenolase with low oxidase activity was induced mainly in the hypocotyl and is possibly concerned with lignin biogenesis, whereas the high-oxidase phenolases present in the cotyledons or induced in leaves may play some role in the protection of the tissue following physical damage.

INTRODUCTION

The roles of phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) and cinnamate 4-hydroxylase (C4H, EC 1.14.13.11) in catalysing the initial reactions in the biosynthesis of lignin [1, 2], flavonoids [3] and chlorogenic acid [4] from phenylalanine have been successfully established by relating increases in their activities to the accumulation of these phenolic products following environmental changes such as illumination, tissue excision or nitrogen starvation [5]. In some cases, a precisely co-ordinated increase in the activities of the two enzymes has been demonstrated [2, 3]. These two enzymes give rise to *p*-hydroxylated phenolics, but many phenolic products show 3,4-dihydroxy substitution, often with partial or complete methylation, knowledge of the enzymes responsible for this hydroxylation pattern is far from complete. Genetic studies have recently been used in detecting enzymes catalysing the 3-hydroxylation of *p*-coumaroyl CoA [6] and the 3′-hydroxylation of naringenin and dihydrokaempferol [7] in the biogenesis of hydroxylated flavonoids in certain species, though their induction in relation to PAL and C4H has still to be demonstrated. The changes in *p*-coumarate 3-hydroxylase (EC 1.14.17.2) and DOPA oxidase activities have been studied here in relation to PAL and C4H to determine whether any metabolic co-ordination between these enzymes might exist.

The conditions under which the phenolase (EC 1.14.18.1) from mature spinach-beet leaves can catalyse effectively the hydroxylation of *p*-coumarate to caffeate have been described [8, 9], with the elimination of the further oxidation of caffeate to caffeoyl quinone [10]. The capacity to catalyse this hydroxylation does not, however, establish the role of the enzyme *in vivo*, and indeed several arguments can be raised against this function. In many tissues phenolase can catalyse *p*-coumarate hydroxylation at more than 100 times the maximum activity of PAL, the enzyme shows only a low specificity for its phenolic substrate [11, 12], it may be thylakoid-bound in green cells away from the other enzymes of phenylpropanoid metabolism [13] and is much less affected than these by seasonal change [14, 15]. However, phenolases with some discrimination for their phenolic substrate have been reported [16] and also a phenolase from *Sorghum* internodes has been described, which shows low *o*-diphenol oxidase activity relative to its hydroxylase activity [17], so reducing the likelihood of further oxidation of the diphenol.

The response of PAL, C4H and *p*C3H activities in spinach-beet to excision and illumination has been examined here. The illumination of young seedlings has been shown to increase *p*C3H co-ordinately with PAL and C4H, the partial purification and properties of a hydroxylase peculiar to illuminated seedlings is described. Some of these results have been outlined earlier [18, 19].

RESULTS

Enzyme changes in excised leaf discs

When excised discs from mature spinach-beet leaves were floated on water at 25°, *p*C3H and DOPA oxidase activities showed a steady increase over 60 hr, whereas the activities of PAL and C4H declined (Fig. 1). Upon illumination immediately after excision, however, PAL

Abbreviations used: PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; *p*C3H, *p*-coumarate 3-hydroxylase.

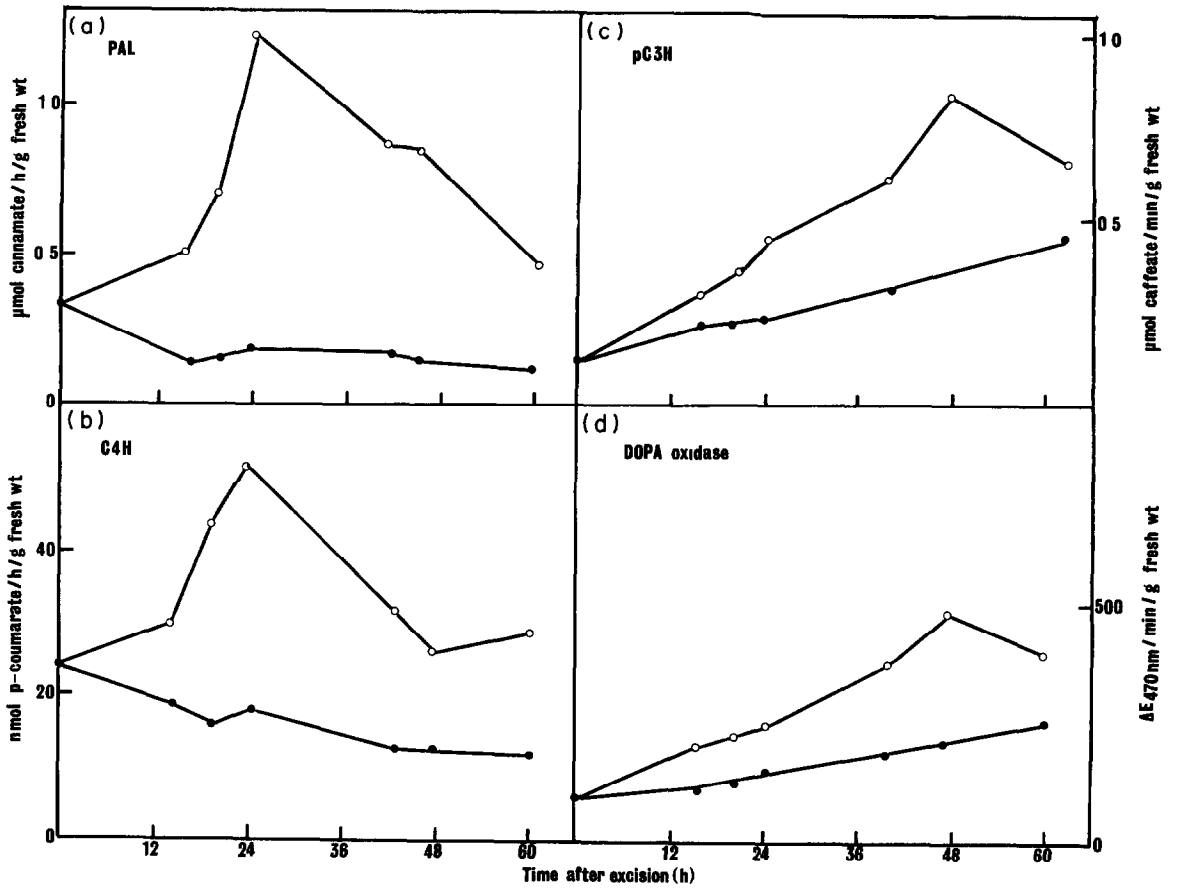

Fig 1 Changes in activities of (a) PAL, (b) C4H, (c) *p*C3H and (d) DOPA oxidase in excised spinach-beet leaf discs under illumination (open symbols) and in darkness (closed symbols)

and C4H increased together following an initial lag period, to reach a peak at 24 hr, they then declined towards the control value There was only a small increase in *p*C3H above the dark controls until 36 hr, after which activity rose to a brief peak at 48 hr, changes in DOPA oxidase followed an almost precisely similar time-course

There was thus no synchronization between PAL and C4H on the one hand and *p*C3H on the other The co-ordination between the increases in *p*C3H and DOPA oxidase implies the induction of phenolase activity observed at a time when the cut surfaces of the leaf became brown

Enzyme changes during seedling growth

Changes in PAL and C4H in the cotyledons and first leaf-pair of spinach-beet seedlings were closely co-ordinated during 32 days of illumination following germination for 6 days in darkness (Fig 2) The same general pattern was observed for *p*C3H and DOPA oxidase in the cotyledons and first leaf pair with peaks of activity possibly rather later than the peaks of PAL and C4H activity, and with additional peaks developing after prolonged illumination The changes suggest increases in phenolase activity, but the imprecise co-ordination between the hydroxylase and oxidase activities may imply changes in a heterogeneous phenolase population with

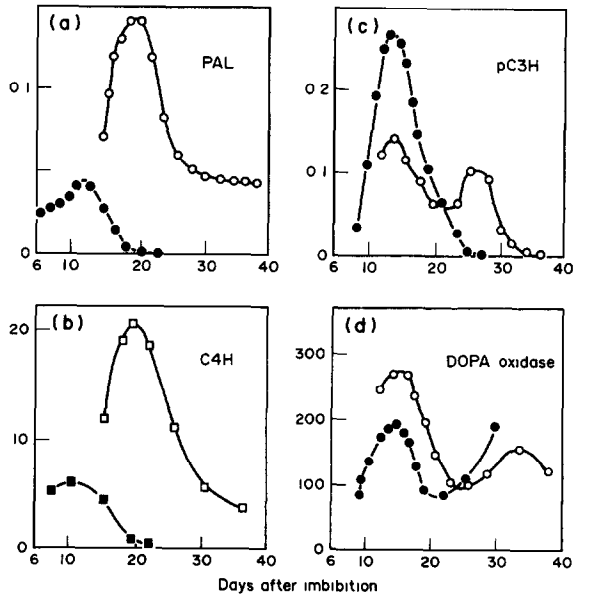

Fig 2 Changes in activities of (a) PAL, (b) C4H, (c) *p*C3H and (d) DOPA oxidase in cotyledons (closed symbols) and first leaves (open symbols) during illumination of spinach-beet seedlings for 32 days under 16 hr light–8 hr dark, after 6 days germination and growth in darkness Abscissa and ordinates as in Fig 1

different hydroxylase–oxidase ratios rather than increases in the activity of any single phenolase

Enzyme responses to illumination

Illumination of etiolated 6-day-old spinach-beet seedlings promoted rapid changes in PAL, C4H and *p*C3H over 24 hr (Fig 3) Changes in PAL and C4H were again closely co-ordinated, and all three enzymes reached a peak at or before 12 hr No changes were observed in seedlings kept in darkness over this period By contrast with PAL and C4H, high activities of *p*C3H were observed in the dark controls but, when these were subtracted from the activities of the illuminated seedlings, the pattern of change in the illuminated seedlings was broadly similar to that of PAL and C4H There were high initial DOPA oxidase and tyrosine hydroxylase activities, but neither showed any change upon illumination The results are consistent with the presence of a phenolase, which did not respond to illumination, and a hydroxylase, which increased in co-ordination with PAL and C4H, this hydroxylase showed very low or no oxidase activity and discriminated between *p*-coumarate and tyrosine as substrates

This conclusion was confirmed by examining the changes induced by illuminating seedlings with red and far-red light (Table 1) At first sight, the closely correlated changes in PAL and C4H do not appear to be reflected by changes in *p*C3H, but if 70% of the initial activity is subtracted from each value the resulting activities show a surprisingly close correlation with the changes in the other two enzymes DOPA oxidase activity did not change The results are again consistent with the development of a hydroxylase with very little or no oxidase activity

Separation and characterization of photo-induced hydroxylase activity

Homogenates of illuminated (6 hr) and non-illuminated etiolated seedlings were purified by ammonium sulphate fractionation, gel filtration and ion-exchange chromatography The increased hydroxylase activity following illumination was found in the 35–70% saturated ammonium sulphate fraction and was eluted in the void volume through Sephadex G-100 (Figs 4A and 4C) The hydroxylase activity retained by the column showed a high hydroxylase–oxidase ratio upon elution (Table 2)

The active eluate from non-illuminated seedlings was fractionated through DEAE-cellulose in a three-step gradient firstly, linearly, from 20 mM disodium orthophosphate–10 mM citrate (pH 5 3) to 100 mM disodium orthophosphate–50 mM citrate (pH 5 3), and then with 200 mM disodium orthophosphate–100 mM citrate (pH 5 3) followed by 1 M sodium chloride The linear gradient gave two peaks of oxidase activity (peaks 1 and 3 respectively, Fig 4B) Subsequent elution with 200 mM

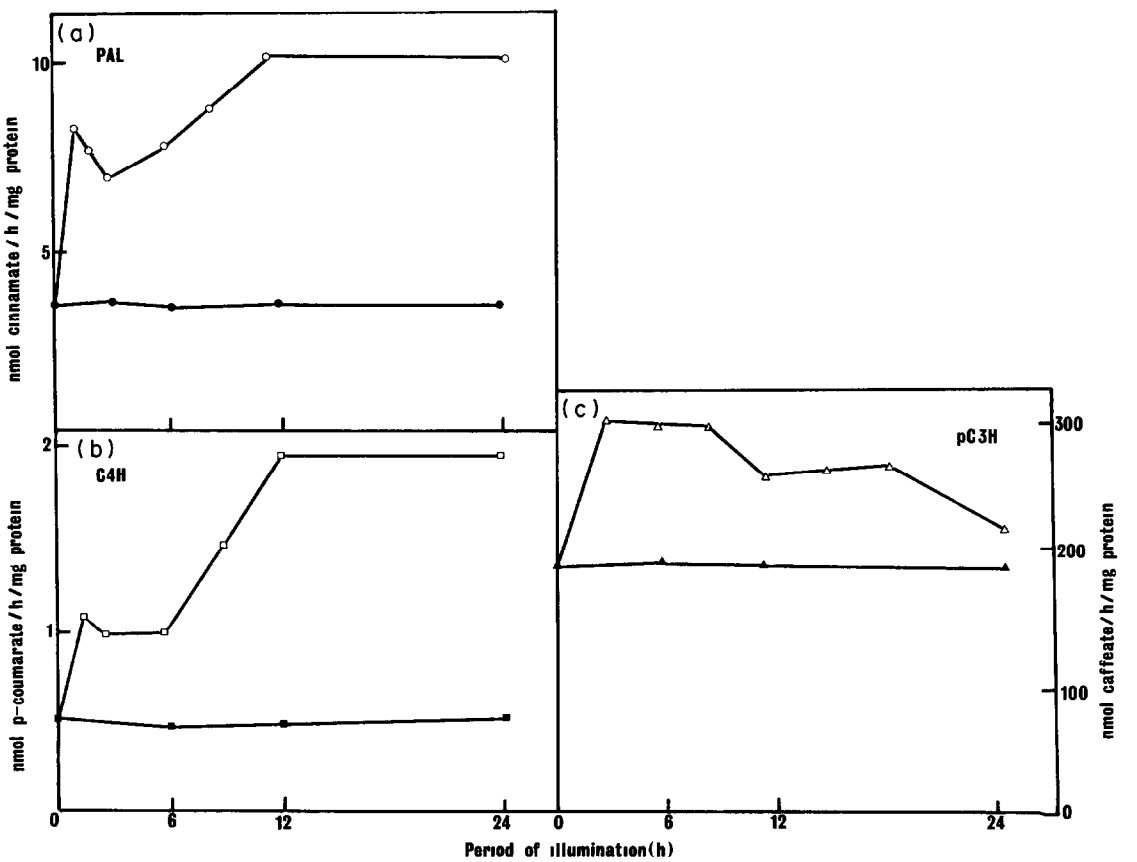

Fig 3 Changes in activities of (a) PAL, (b) C4H and (c) *p*C3H during illumination of 6-day dark-grown spinach-beet seedlings (open symbols) and in seedlings kept in darkness (closed symbols)

Table 1 Effect of red/far-red light treatments on enzyme levels in etiolated spinach-beet seedlings

Treatment	Enzyme activities				
	Phenylalanine ammonia-lyase (nmol/hr/mg protein)	Cinnamate 4-hydroxylase (nmol/hr/mg protein)	*p*-Coumarate 3-hydroxylase (nmol/hr/mg protein)		DOPA oxidase (ΔE_{470}/min/mg protein)
			1	2*	
Darkness	3 25(1 0)	0 42(1 0)	317(1 0)	87(1 0)	20 6
Red (15 min)–darkness	9 77(3 0)	1 12(2 6)	481(1 5)	251(2 9)	21 4
Red (15 min)–far-red (5 min)–darkness	5 46(1 7)	0 57(1 4)	363(1 1)	133(1 5)	20 0
Far-red (5 min)–darkness	5 52(1 7)	0 63(1 5)	370(1 1)	140(1 6)	20 4
Red	10 02(3 1)	1 43(3 4)	514(1 6)	284(3 3)	20 6

6-Day-old dark-grown seedlings were exposed to the conditions indicated for a total period of 3 hr before assay Figures in parentheses indicate activity of each enzyme relative to its dark control

*The data in column 2 are those in column 1 after deduction of 230 nmol/hr/mg protein

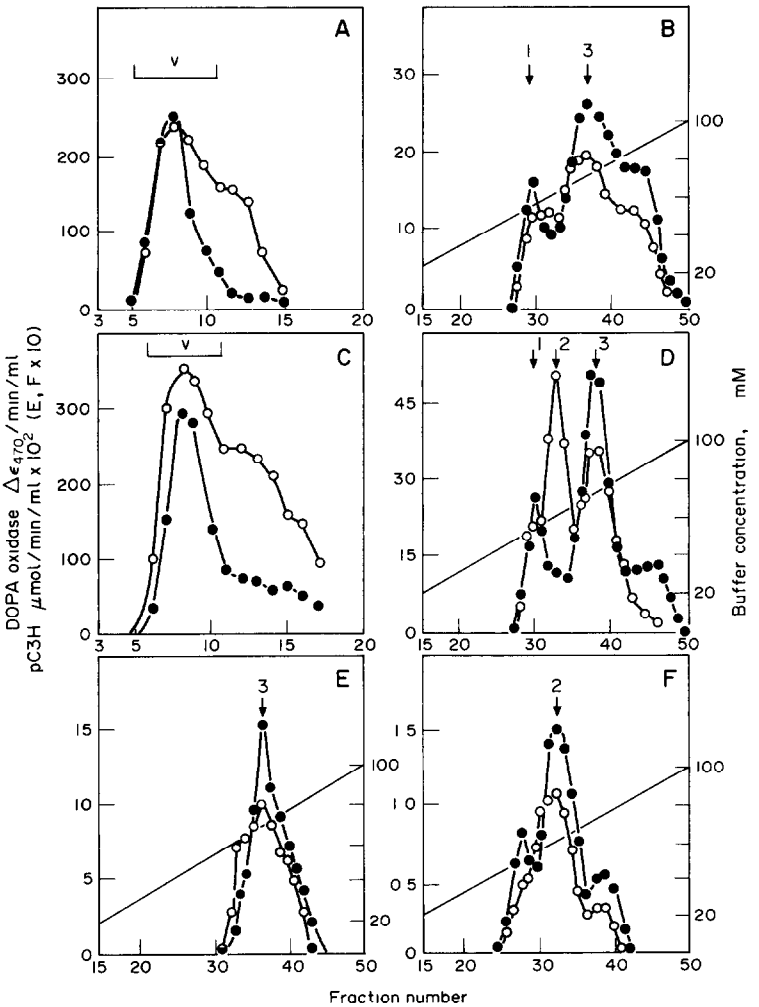

Fig 4 Elution profiles of *p*C3H (○) and DOPA oxidase (●) prepared from dark-grown seedlings (30 g) on (A) Sephadex G-100 followed by chromatography of fractions 6–10 on (B) DEAE-cellulose, and from dark-grown seedlings (38 g) illuminated for 6 hr on (C) Sephadex G-100 followed by chromatography of fractions 6–10 (D) and 11–17 (E) on DEAE-cellulose (F) Chromatography of fractions 31–35 (peak 2) from (D) on DEAE-cellulose Numbers refer to peaks discussed in the text Elution as described in the Experimental

Table 2 Hydroxylase and oxidase activities in fractions from illuminated seedlings and their sensitivity to phenolase inhibitors

Fraction	Total activity		Recovery (%)		Ratio		% inhibition			
							0·1 mM diethyl dithiocarbamate		0·1 mM salicoyl hydroxamate	
	*p*C3H	DOPA oxidase	*p*C3H	DOPA oxidase	*p*C3H/DOPA oxidase	Tyrosine hydroxylase	*p*C3H	DOPA oxidase	*p*C3H	DOPA oxidase
Crude	44·4	2106	100	100	—	—	—	—	—	—
35–70% $(NH_4)_2SO_4$	39·9	842	90	40	—	—	—	—	—	—
Fractions 6–10 from Sephadex column (void)	27·9	1980	63	94	—	—	10	60	20	87
Peak 1	2·3	210	5	10	0·014	35·2	100	90	100	95
2	8·9	110	20	5	0·011	1·2	100	95	100	100
3	10·6	1185	22	56	0·081	0	63	92	90	95
4	1·7	198	4	9	0·009	43·2	100	98	57	89
5	2·2	204	5	10	0·011	50·4	40	85	50	80
Low MW* (fractions 11–17 from Sephadex column)	19·5	294	44	14	0·066	2·5	100	100	95	90
2(2)	4·7	46	11	2	0·102	0	100	100	100	100

Enzyme activities are given for fractions described and depicted in Figs 4(C)–4(F), and in Fig 5 *p*C3H and tyrosine 3-hydroxylase activities are shown as μmol/hr and DOPA oxidase activities as ΔE_{470}/min *p*C3H and DOPA oxidase activities of all fractions were completely inhibited by 1 mM diethyldithiocarbamate and 1 mM salicoyl hydroxamate, but not by 1 mM 2,2′-bipyridyl

disodium orthophosphate–100 mM citrate (pH 5 3) followed by 1 M sodium chloride gave two peaks (not shown), 4 and 5, respectively Each peak correlated with peaks of hydroxylase activity, but the ratios of the two activities in the peaks varied (Table 2) Fractionation of the eluate from illuminated seedlings gave the same four peaks but also an additional peak (peak 2), which accounted for all of the additional hydroxylase activity (Fig 4D) The oxidase retained by Sephadex G-100 and subsequently eluted emerged from DEAE-cellulose as a single peak corresponding to peak 3 (Fig 4E)

Each fraction was subjected to polyacrylamide gel electrophoresis, and stained for oxidase activity (Fig 5) Complex patterns were observed in peaks 3 and 4 and in the low MW fraction retained by Sephadex G-100 Peaks 1 and 2 were barely distinguishable and appeared to consist of three components, MWs 40 000, 80 000 and above 100 000, respectively No hydroxylase activity was detected on gels by the methods employed

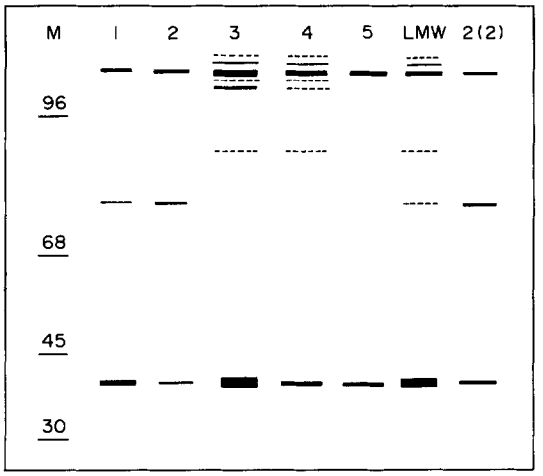

Fig 5 Polyacrylamide (10%) gel electrophoresis of peak fractions partially denatured at room temperature M, Marker proteins with MWs ($\times 10^{-3}$), 1–5, peak fractions (see Fig 4D and text), LMW, low MW fraction from Sephadex G-100 after chromatography on DEAE-cellulose, 2(2), peak 2 after rechromatography (see Fig 4F)

Each peak showed identical K_m values for DOPA (2 35 mM) and *p*-coumarate (0 27 mM) Three of the peaks (1, 4 and 5) also catalysed the hydroxylation of tyrosine, but the fraction induced by illumination (peak 2) was active only with *p*-coumarate The hydroxylase activity of each peak was competitively inhibited by salicoyl hydroxamate and benzoyl hydroxamate [20], and non-competitively by the specific copper chelator, diethyldithiocarbamate, all were unaffected by 1 mM 2,2'-bipyridyl, showing that the hydroxylation was not activated by Fe^{2+} (Table 2)

The similarity of response of the peaks to these different reagents and their similar electrophoretic patterns suggest that they are multiple forms of the same enzyme rather than isoenzymes This multiplicity could be demonstrated only if sufficient ascorbate was added to the homogenization medium to prevent irreversible binding to DEAE-cellulose and if ammonium sulphate was removed completely by gel filtration, since ammonium sulphate, although necessary for maximum hydroxylase activity [8], inhibits oxidase activity and is responsible for the apparently excessive oxidase recovery after gel filtration With these precautions, the hydroxylase and oxidase activities of the original homogenate are accounted for in the subsequent procedure (Table 2)

Further chromatography of the light-induced hydroxylase activity (peak 2) through DEAE-cellulose gave a single peak (Fig 4F), again with a high hydroxylase–oxidase ratio (Table 2) The preparation had catechol oxidase activity but did not hydroxylate tyrosine Gel electrophoresis showed the same oxidase pattern, with significantly greater proportions of the 80 000 MW form (Fig 5)

Since most of the light-induced hydroxylase activity had been found in the seedling hypocotyls, extracts from the hypocotyls and cotyledons of illuminated seedlings were subjected to the same fractionation procedures and the elution patterns from DEAE-cellulose compared (Table 3) The total hydroxylase activities were closely related to the weights of hypocotyl and cotyledon extracted, by comparison, the oxidase activities were similar

Table 3 Hydroxylase and oxidase activities in fractions from hypocotyls and cotyledons of illuminated seedlings

	Hypocotyls			Cotyledons		
	Total activity		Ratio	Total activity		Ratio
Fraction	*p*C3H	DOPA oxidase	*p*C3H/oxidase	*p*C3H	DOPA oxidase	*p*C3H/oxidase
Peak 1	4 8	71	0 067	5 8	74	0 078
Peak 2	8 4	32	0 266	2 3	22	0 104
Peak 3	8 2	189	0 043	4 6	375	0 012
Peak 4	4 3	168	0 025	1 2	70	0 017
Peak 5	5 2	183	0 028	1 1	77	0 014
% recovery of activity	109	65	—	88	58	—

Dark-grown seedlings (52 g), illuminated for 6 hr, were separated into hypocotyls (36 g) and cotyledons (15 g) The homogenates were fractionated as described in the Experimental and Fig 4 *p*C3H activity is given as μmol/hr and DOPA oxidase activity as E_{470}/min

despite this difference in weight Peak 2 again showed an exceptionally high hydroxylase–oxidase ratio, especially in the hypocotyls This peak was the major hydroxylase peak in the hypocotyls, and the hypocotyl peak accounted for 80% of the total activity of peak 2 in hypocotyls and cotyledons By comparison, peak 3 carried 60% of the oxidase activity in the cotyledons and was also the major oxidase peak in the hypocotyls, peaks 4 and 5 were substantially less active in the cotyledons but from both hypocotyls and cotyledons, their hydroxylase–oxidase ratios were similar to those of the corresponding peak 3 It is concluded that the hydroxylase activity of peak 2 is probably the significant enzyme in light-stimulated hydroxylation, while the oxidase activity of peak 3, together with peaks 4 and 5, is probably more important than its capacity to catalyse hydroxylation

DISCUSSION

Two different patterns of response by *p*C3H and DOPA oxidase to environmental treatment are reported here In excised leaf discs and during seedling growth, there appears to be a virtually co-ordinate increase in the two activities These may be simultaneous with changes in PAL and C4H, as in the cotyledons and first leaves of illuminated seedlings, or subsequent to these changes Neither give reason to believe that the increased phenolase activity takes part in biosynthetic processes and, especially in the leaf discs, it seems as likely that the enzyme provides an armoury of oxidative, and perhaps also of hydroxylative, activity in the green parts against surface damage, whether by physical agents [21] or by predators and pathogens [22, 23] This is supported by the development of browning at the cut surfaces of the leaf discs as the phenolase develops [19]

The second pattern is that observed in seedlings subjected to white or red light Here the changes in *p*C3H are correlated with changes in PAL and C4H, but no changes in DOPA oxidase activity were found These changes take place against a high phenolase background, proportionately higher in the cotyledons than the hypocotyls Much of the *p*C3H response is found in the hypocotyls, and it may be significant that fractionation studies have found the induced hydroxylase peak to be four times as active in the hypocotyl as in the cotyledons Comparison with the response of etiolated pea seedlings to illumination suggests that this enzyme is concerned with lignification, in the stem apices of pea seedlings, 80% of the lignification induced by light takes place in the epicotyls, compared with the apical buds, with a corresponding distribution of the enzymes converting caffeic acid to coniferyl alcohol and of cell wall peroxidase [2] These latter enzymes are already present in etiolated pea seedlings [2] and soya cells [1] and do not increase in activity during lignin production, they may be confined to specific cells able to undergo differentiation upon induction of the earlier enzymes It seems likely that *p*C3H is associated with PAL and C4H in a similar process in spinach beet seedlings, even though some reservation might be expressed because of its relatively high activity, before and subsequent to illumination

These results invite comparison with phenolase activity in the first internodes of *Sorghum bicolor* seedlings [17], which produce cyanidin glycosides upon illumination [24] A low MW phenolase, which catalysed the hydroxylation of *p*-coumarate with only weak diphenol oxidase activity, was separated by Sephadex G-100 gel filtration from higher MW forms with high diphenol oxidase activities, one of which could also catalyse the hydroxylation, the properties of these higher MW forms were generally similar to those of the spinach-beet phenolase The internode hydroxylase, by comparison with that from *Sorghum* leaves, did not appear to aggregate and showed no increase in oxidase activity on purification through a hydroxyapatite column [25] From spinach-beet seedlings, however, the low MW forms, showing a major 40000 MW band, possessed relatively low hydroxylase activity, and the photoinduced hydroxylase showed bands of similar intensity corresponding to MWs of 40000, 80000 and 120000 (or possibly, 160000), respectively, on PAGE A precise comparison between the two systems is difficult because no observations on phenolase activity in unilluminated *Sorghum* internodes have been described

The presence of multiple forms of polyphenol oxidase in spinach leaves has been demonstrated for both thylakoid-bound and stromal enzymes [26, 27] The proportions of these different forms varies with age but no evidence that the production of any of them depends upon illumination has been reported Furthermore, they are reported not to show hydroxylase activity with the exception of a single, weak form present mainly in younger primary leaves Forms similar to peaks 1 and 3 have been isolated from membranes and separated by gel filtration and DEAE-cellulose chromatography and have been claimed to correspond to tetramer and monomer, respectively [28], but gel electrophoresis of the forms reported here show distinct heterogeneity It remains to be established whether peak 2 reported here is in any way similar to the only form capable of catalysing the hydroxylation of monophenols isolated from spinach chloroplasts [26], and further physical analysis is necessary to determine the essential difference between the enzymes of peaks 1 and 2, which, although having a similar composite structure, differ widely in their relative capacities for hydroxylating monophenols

The photoinduced increase in *p*C3H activity thus appears to be due to an increase in a copper oxygenase with only limited oxidase activity The properties of this enzyme show that it is not fundamentally different from the other phenolases present, but is probably another form with a different type of aggregation or conformation which facilitates hydroxylation with suppression of further oxidation of the *o*-diphenol formed

EXPERIMENTAL

Plant material and growth conditions Seeds of spinach-beet (*Beta vulgaris* L cv) were germinated in vermiculite in growth cabinets at 25°, either in darkness or under a 16 hr light–8 hr dark regime

White light was provided by fluorescent tubes, giving an intensity of 53×10^3 ergs/cm^2/sec at soil level Red light was provided by fluorescent tubes filtered through one layer of No 1 and one layer of No 6 Cinemoid (Rank Strand Electric), the transmitted light having wavelengths between 570 and 710 nm ($\lambda_{max} = 630$ nm) with a total flux of 320 ergs/cm^2/sec Far-red light was provided by using an FRF 700 Plexiglass filter (Westlake Plastics, Lenni Mills, PA, U S A) with a wavelength spread from 710 to 750 nm at an intensity of 450 ergs/cm^2/sec at soil level The growth room was illuminated only by a green safelight, under which enzyme extractions were also carried out

Discs were excised from mature leaves of plants, grown outdoors, with a 7 mm cork borer and floated on H_2O containing streptomycin (25 mg/l) and $CaSO_4$ (50 mg/l) The discs were incubated at 25° either in darkness or under the white light source

Enzyme assays When multiple enzyme assays were performed on the same tissue, each enzyme was extracted from separate samples of the same batch by the procedures described here If the enzymes were assayed in separate expts, PAL was used as the reference enzyme, because of its reproducibility Each expt was repeated × 3, with no significant variation in the patterns of change with age of seedling or with the period of illumination

(1) PAL was extracted by homogenizing the tissue in 50 mM Tris–HCl buffer (pH 8 5) with insoluble PVP (10% wt of plant material) After squeezing through muslin, the homogenate was centrifuged at 2500 *g* for 30 min, and the supernatant then assayed for PAL activity spectrophotometrically [29]

(2) C4H was extracted by homogenizing in 50 mM KH_2PO_4–K_2HPO_4 (pH 7 5) containing 1 mM EDTA and 20 mM mercaptoethanol After it had been squeezed through muslin, the extract was centrifuged at 1200 *g* for 30 min and the supernatant assayed for C4H radiometrically, using [2-^{14}C]cinnamate as substrate [30]

(3) *p*C3H activity was extracted in 10 mM Na_2HPO_4–5 mM citric acid buffer (pH 5 3) The homogenate was squeezed through muslin and centrifuged at 2000 *g* for 2 min *p*C3H was assayed colorimetrically [8], using ascorbate as electron donor Tyrosine hydroxylation was determined with the substitution of 1 67 mM tyrosine for *p*-coumarate and measuring the *A* at 385 nm

o-Diphenol oxidase activity of this extract was determined either spectrophotometrically using DOPA as substrate [31], or with the O_2 electrode using 4-methylcatechol as substrate The reaction mixture contained 4-methylcatechol (1 μmol) and ascorbate (10 μmol) with $(NH_4)_2SO_4$ (1 5 mmol), Na_2HPO_4 (100 μmol)–citric acid (50 μmol) buffer, pH 5 3, and extract in a total vol of 3 0 ml

Protein was estimated by the Folin [32] or Coomassie blue [33] methods

Partial purification of phenolase Seedlings, whether dark-grown or grown in darkness followed by exposure to continuous white light for 6 hr, were homogenized in Na_2HPO_4 (50 mM)–citric acid (25 mM) buffer, pH 5 3, containing 20 mM ascorbate, sufficient to give a 20% (w/v) homogenate This was centrifuged at 2000 *g* for 90 sec and the supernatant soln fractionated with $(NH_4)_2SO_4$ The fraction sedimented by 35–70% satd $(NH_4)_2SO_4$ was gel-filtered through a column (25 × 2 cm) of Sephadex G-100 equilibrated with 1 mM Na_2HPO_4–KH_2PO_4 buffer, pH 7 2, and suitable fractions were then passed through columns (20 × 1 cm) of DEAE-cellulose, which had been equilibrated with 1 mM Na_2HPO_4–KH_2PO_4 buffer, pH 7 2 Fractions were eluted with a linear gradient between 20 mM KH_2PO_4–10 mM citrate buffer (pH 5 3) and 100 mM KH_2PO_4–50 mM citrate buffer (pH 5 3), followed successively by 20 ml 200 mM KH_2PO_4–100 mM citrate buffer (pH 5 3) and 20 ml 1 0 M NaCl When the gel-filtration stage was omitted, the $(NH_4)_2SO_4$ fraction was dialysed for at least 5 hr against 1 mM Na_2HPO_4–KH_2PO_4 buffer (pH 7 2) before layering it on the DEAE-cellulose column

Gel electrophoresis SDS-polyacrylamide slab-gel electrophoresis was carried out by the method of Laemmli [34], modified by Studier [35] Fractions were lyophilized before partial denaturation at room temp in sample buffer (0 08 M Tris–HCl, pH 6 8, 10% glycerol, 2% SDS, 50 mM dithiothreitol, 0 0005% bromophenol blue) Phenolase bands were visualized by incubating the gel in 50 mM Na_2HPO_4–25 mM citric acid, pH 5 3, containing 10 mM DOPA at room temp Hydroxylase activity was tested by incubating the gel with 3 3 mM *p*-coumarate and 3 3 mM ascorbate in 100 mM Na_2HPO_4–50 mM citric acid, pH 5 3, containing 0 5 M $(NH_4)_2SO_4$, and detecting the caffeic acid produced either by colour production [8] or by UV fluorescence

Acknowledgements—G P B thanks the Science Research Council for a Research Studentship held during the period when much of this work was carried out, and to Professor D H Northcote for his encouragement and facilities to complete the work during the tenure of a S R C Research Fellowship We are grateful to Dr P R Rich for the gift of salicoyl and benzoyl hydroxamates

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