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EFFECT OF AUXIN ON THE METABOLISM OF MEVALONIC ACID IN SUSPENSION-CULTURED CARROT CELLS

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Key Word Index—*Daucus carota*; Umbelliferae; suspension culture; HMG-CoA reductase; mevalonate kinase; 2,4-dichlorophenoxyacetic acid.

Abstract—The rate of incorporation of [^{14}C]mevalonate into carotenoid and steroid fractions in suspension-cultured carrot cells decreased markedly after 2,4-dichlorophenoxyacetic acid was removed from the medium. In parallel to this change, the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase in a microsomal fraction was reduced to *ca* 33% of the control value, while that of a particulate fraction showed no significant change. The activities of mevalonate activating enzymes remained unchanged after auxin deprivation.

INTRODUCTION

Previous studies have shown that the production of carotenoid and steroid in suspension-cultured carrot cells is dependent on the presence of 2,4-dichlorophenoxyacetic acid (2,4-D) as auxin [1]. Biosynthesis of phenolic acids in the same strain of carrot cells is, on the other hand, depressed by 2,4-D [2]. In many plant cell cultures, auxin has a marked influence on the production of secondary metabolites [3, 4]. In spite of its important regulatory role, very few papers dealing with the change in enzyme level as affected by auxin have been published, though there is evidence that certain enzymes are activated [5] or inactivated [6] by it. As the first step to elucidate the mechanism by which the synthesis of carotenoid and steroid is regulated by auxin, we studied the change in the enzyme activities in mevalonate (MVA) metabolism in carrot cells when 2,4-D is removed from the culture medium.

RESULTS AND DISCUSSION

Carrot cells used in this experiment accumulate large amounts of carotenoids and steroids, namely lycopene and β-carotene and stigmasterol, campesterol and sitosterol [1]. Total contents of carotenoids and steroids in the cells cultured in an ordinary medium containing 1 mg/l. 2,4-D were *ca* 0.9 and 4.5 mg/g dry wt, respectively [1]. When the cells were transferred to a medium lacking 2,4-D, the contents of both metabolites decreased rapidly although the growth rate was almost unaffected for *ca* 1 week. The culture turned from reddish-yellow to pale yellow within a few days. Table 1 shows the rate of incorporation of [^{14}C]MVA into carotenoid and steroid fractions of the carrot cells. After 4 days cultivation in the absence of 2,4-D, the rate decreased markedly, especially in the steroid fraction. The activity of 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase) was measured in those cells (Table 2). The enzyme activity was distributed both in particulate (10000 *g* pellet) and microsomal (105000 *g* pellet) fractions. On removing the auxin, the enzyme activity in the microsomal fraction declined so that at day 4 the activity dropped to a level of *ca* 33% of the control. On the other hand, the activity in the particulate fraction showed only a slight change after the treatment.

MVA is considered to be the key intermediate in the biosynthesis of isoprenoids and it is generally accepted that HMG-CoA reductase is an important control step in steroid synthesis in mammalian cells [7]. Very few reports on the enzyme from higher plants, however, have been published. Suzuki and Uritani have found that in sweet

Table 1. Incorporation of [^{14}C]MVA into carotenoid and steroid fractions of carrot cells

		MVA incorporated (nmol/g fr. wt)	
Fractions		Control (+2,4-D)	−2,4-D
Expt 1	Carotenoid	2.3	0.9
	Steroid	2.5	0.5
Expt 2	Carotenoid	2.0	0.5
	Steroid	2.4	0.4

Cells were precultured in the presence of 1 mg/l. 2,4-D, then transferred to a fresh medium with or without 2,4-D and cultured for 4 days before the experiment.

Table 2. HMG-CoA reductase activity in carrot cells

		MVA synthesized (nmol/hr·mg protein)	
Fractions		Control (+2,4-D)	−2,4-D
Expt 1	10000 *g* pellet	2.3	1.8
	105000 *g* pellet	6.9	2.1
Expt 2	10000 *g* pellet	2.1	1.6
	105000 *g* pellet	8.6	2.9

Culture conditions were the same as in Table 1.

potato root the enzyme is localized in mitochondria but not in microsomal and soluble fractions [8]. In diseased tissues, however, the enzyme activity was found also in the microsomal fraction where it participates in the synthesis of furanoterpenes which are produced when the tissue is infected with fungi. Their results suggest that the enzyme in mitochondria is constitutive whilst that in microsomal fraction varies according to environmental stress. Our results are in consistent with this. The decrease in the rate of formation of carotenoid and steroid after the removal of auxin from the culture medium was accompanied by a marked decrease in the enzyme activity of microsomal fraction, whereas the activity in the particulate fraction showed no significant change.

MVA is metabolized successively to mevalonate 5-phosphate (MVA-5-P), mevalonate 5-pyrophosphate (MVA-5-PP) and isopentenyl pyrophosphate by mevalonate-activating enzymes. To determine the activities of these enzymes in carrot cells, [^{14}C]MVA was incubated with a particulate or a soluble (105 000 g supernatant) fraction and the products were analysed by PC. Radioactivity was found in regions corresponding to MVA-5-P and MVA-5-PP. The presence of isopentenyl pyrophosphate was not confirmed. Table 3 shows the distribution of these phosphorylated intermediates in the products after incubation with enzyme preparation obtained from cells cultured in the presence or absence of 2,4-D. The activity was found both in soluble and particulate fractions. The experimental values fluctuated considerably but the results suggest that the activities of these enzymes were relatively unchanged after auxin deprivation. A similar result was obtained when the MVA kinase activity was measured by a spectrophotometric method [9] with partially purified preparations (data not shown). It seems that these enzymes are not rate-limiting in the biosynthesis of isoprenoids because their specific activities were much higher than that of HMG-CoA reductase.

These results show that amongst the enzymes directly concerned in MVA metabolism, only HMG-CoA reductase bound to the microsomal fraction was influenced by the presence or absence of auxin and suggest that the enzyme took part in the accumulation of large amounts of carotenoids and steroids in this cell. This does not necessarily mean that HMG-CoA reductase is the sole step in the regulation of the syntheses of carotenoid and steroid, and there may be control at later stages in the biosyntheses.

EXPERIMENTAL

Plant cells and cultivation. Carrot cells of strain GD3 [1], which had been derived from a root of red carrot (*Daucus carota* L., cv Kintoki), were used. The cells were cultured in the synthetic medium of Murashige and Skoog [10] supplemented with 3% sucrose and 1 mg/l. 2,4-D. The culture was maintained in 500 ml flasks containing 100 ml of the liquid medium and the flasks were agitated on a reciprocal shaker at 130 strokes/min at 26° under dim light. When the cells were transferred to a medium lacking 2,4-D a 5-day-old culture was centrifuged for 2 min at 500 g. The cells were washed twice with auxin-free medium, then resuspended in a medium of the same composition but containing no auxin.

Incorporation of [^{14}C]*MVA into carotenoid and steroid fractions.* DL-[2-^{14}C]Mevalonolactone (MVL, New England Nuclear, Boston) was converted to K-MVA by incubation with KOH according to ref. [9]. The sp act. was adjusted to the required value by the addition of unlabelled compound. Carrot cells in the growth medium were incubated with [^{14}C]MVA (sp. act. 3.2 μCi/μmol) for 3 hr, then washed thoroughly with H_2O on a glass filter. Carotenoids and steroids were extracted with Me_2CO as described previously [1]. The extract was chromatographed on Si gel with 5% Me_2CO in $CHCl_3$. Carotenoid and steroid spots (R_f 0.88–0.95 and 0.48–0.60, respectively) were scraped off and eluted with Me_2CO. The radioactivity of each eluate was determined by a scintillation spectrometer using a commercial scintillation mixture, ACS II.

Cell fractionation. Carrot cells were collected on a glass filter and washed with KPi buffer (pH 7.5, 100 mM) containing 0.5 M sucrose, 10 mM EDTA, 10 mM 2-mercaptoethanol and 1% bovine serum albumin, and ground in a mortar for 2 min. The homogenate was centrifuged at 900 g for 10 min to remove cell debris and undisrupted cells. The supernatant was then fractionated by differential centrifugation at 10 000 g for 20 min and then 105 000 g for 60 min. The protein content of each fraction was determined according to ref. [11].

Enzyme assay. The assay of HMG-CoA reductase was carried out according to ref. [12]. The reaction mixture (final vol. 250 μl) contained 12.5 μmol KPi buffer (pH 7.5), 3 μmol EDTA, 6.5 μmol dithiothreitol, 2 μmol $NADP^+$, 2 μmol glucose 6-phosphate (G-6-P), 0.1 unit G-6-P dehydrogenase, 4 μCi [^{14}C]HMG-CoA (52.6 μCi/μmol, New England Nuclear) and enzyme preparation. After incubation for 60 min at 30°, the reaction was stopped by the addition of 10 μl 10 N NaOH. The mixture was then acidified by adding 20 μl 10 N HCl and incubated for 30 min at 37° to permit MVA to lactonize. After centrifugation, a portion (100 μl) was applied to a Si gel plate and developed with C_6H_6–Me_2CO

Table 3. MVA activating enzymes in carrot cells

Fractions		MVA incorporated into phosphorylated intermediates (nmol/hr·mg protein)					
		Control (+2,4-D)			−2,4-D		
		MVA-P	MVA-PP	Sum	MVA-P	MVA-PP	Sum
Expt 1	10 000 g pellet	24.8	64.5	89.3	20.5	49.8	70.3
	105 000 g supernatant	4.3	9.9	14.2	5.5	10.4	15.9
Expt 2	10 000 g pellet	19.5	43.6	63.1	24.9	63.7	88.9
	105 000 g supernatant	4.0	9.5	13.5	2.8	12.8	15.1
Expt 3	10 000 g pellet	16.9	54.1	71.0	24.2	57.8	95.7
	105 000 g supernatant	4.3	15.6	19.9	2.8	8.6	11.4

Culture conditions were the same as in Table 1.

(1:1). The region of MVL was scraped off and the radioactivity determined.

MVA kinase activity was assayed according to ref. [9] with some modifications. Reaction mixture (final vol. 1 ml) contained 50 μmol KPi buffer (pH 7.0), 5 μmol ATP, 5 μmol $MgCl_2$, 0.4 μCi [^{14}C]MVA (sp. act. 0.4 μCi/μmol) and enzyme preparation. Incubation was carried out at 30° for 60 min and terminated by heating at 80°. After centrifugation, the supernatant was freeze-dried and redissolved in 0.2 ml H_2O. A portion (50 μl) of the soln was subjected to PC with *n*-BuOH–HCO_2H–H_2O (77:10:13) [13]. Developed paper was dissected into strips of 5 mm length and radioactive compounds were eluted with H_2O. Identification of the labelled compounds was carried out according to ref. [13] by comparing the chromatographic behaviour after acid or alkaline hydrolysis.

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