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# FORMATION OF PHENOLIC ACID IN CARROT CELLS IN SUSPENSION CULTURES

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**Key Word Index**—*Daucus carota*; Umbelliferae; carrot; suspension culture; biosynthesis; phenolic acid; cinnamic derivatives.

**Abstract**—The formation of phenolic acid in carrot cells grown in suspension culture was examined in relation to cell growth and the concentration of 2,4-dichlorophenoxyacetic acid (2,4-D). Cells multiplied and grew through logarithmic, linear and stationary phases. At all these phases, caffeic, ferulic and *p*-hydroxybenzoic acids were detected. Biosynthesis of these acids was higher in the early logarithmic phase than at any other phase and rapidly declined in the stationary phase. By lowering the concentration of 2,4-D, these organic acids, and the activity of phenylalanine ammonia-lyase were markedly increased.

## INTRODUCTION

The recent progress in tissue culture has given us several approaches for the study of secondary metabolism in plants [1]. In the preceding papers [2–6], we have reported that carrot callus tissue shows higher synthetic abilities for phenolics and carotenoids than the normal root tissue. It was also found that carotenoid formation was in parallel with the rate of growth and was controlled by the auxin (2,4-D) [5]. In the present paper, the levels of cinnamic and benzoic acid derivatives were further studied on the same carrot cell line in relation to the cell growth and the concentration of 2,4-D.

## RESULTS AND DISCUSSION

After about $2 \times 10^5$ cells were transferred into 150 ml of a fresh medium, cells multiplied logarithmically for 8 days and then linearly for about 3 days before the culture entered into stationary phase. The cell number was then $80 \times 10^5$/ml. In order to examine how the phenolic acids behave at these growth phases, cells were harvested from 3-day-culture (early logarithmic phase), 6-day-culture (late logarithmic phase), 10-day-culture (linear phase) and 14-day-culture (stationary phase). At all of the above phases, three phenolic acids, caffeic, ferulic and *p*-hydroxybenzoic acids were always present (Table 1). The highest contents of these acids were found in 3-day-culture when cells actively divide and show the rapid synthesis or turnover of protein (Table 2). These acids decreased in 6-day-culture but again slightly increased in 10-day-culture. After the cell growth ceased, at stationary phase, the contents of cinnamic deriva-

Table 1. The content of phenolic acid in a carrot cell

| Day of culture | Content (pg/$10^3$ cells) Caffeic acid | Ferulic acid | *p*-Hydroxybenzoic acid |
|---|---|---|---|
| 3 | 276 | 43 | 66 |
| 6 | 138 | 24 | 29 |
| 10 | 195 | 28 | 38 |
| 14 | 59 | 11 | 46 |

Table 2. The incorporation of phenylalanine-[U-$^{14}$C] into protein in carrot cells

| Day of culture | Specific activity (cpm/mg protein × $10^3$) |
|---|---|
| 3 | 185 |
| 6 | 31 |
| 6* | 34 |
| 10 | 12 |
| 14 | 8 |

* Result from cells grown in 0·05 ppm 2,4-D.

Table 3. The incorporation of phenylalanine-[U-$^{14}$C] into phenolic acid in carrot cells

| Day of culture | cpm/$10^6$ cells | | | Specific activity (cpm/nmol) | | |
|---|---|---|---|---|---|---|
| | Caffeic acid | Ferulic acid | *p*-Hydroxybenzoic acid | Caffeic acid | Ferulic acid | *p*-Hydroxybenzoic acid |
| 3 | 956 | 61 | 47 | 125 | 55 | 20 |
| 6 | 130 | 10 | 2 | 34 | 16 | 2 |
| 10 | 106 | 5 | 11 | 20 | 6 | 8 |
| 14 | 5 | 1 | 3 | 3 | 3 | 2 |

Table 4. The effect of concentration of 2,4-D on the content of cinnamic derivatives in carrot cells

| Concentration of 2,4-D (ppm) | Content | | | |
|---|---|---|---|---|
| | μg/g dry wt | | pg/$10^3$ cells | |
| | Caffeic acid | Ferulic acid | Caffeic acid | Ferulic acid |
| 0·05 | 836 | 234 | 2790 | 78 |
| 0·5 | 60 | 10 | 110 | 18 |
| 1·0 | 49 | 9 | 103 | 17 |
| 5·0 | 38 | 16 | 72 | 31 |
| 10·0 | 23 | 27 | 30 | 35 |

tives caffeic and ferulic acids drastically declined whereas that of *p*-hydroxybenzoic acid still increased. On the other hand, the incorporation of phenylalanine-[U-$^{14}$C] into the phenolic acids was much higher in the 3-day-culture than at any other time. Radioactivity in cinnamic derivatives progressively decreased during the phases of declining growth although that in *p*-hydroxybenzoic acid increased slightly in the 10-day-culture. In 14-day-cultures, incorporation did not occur to a significant extent (Table 3). The biosynthetic activity of phenolic acid is therefore correlated with the cell growth phase. The increase in the content of *p*-hydroxybenzoic acid after 6 day's culture may result from the degradation and/or the β-oxidation of $C_3$-side chain in cinnamic derivative.

The effect of auxin (2,4-D) on the formation of cinnamic derivative was examined in a 6-day-culture (Table 4). When cells were grown in a medium containing 0·05 ppm 2,4-D, the content of caffeic acid rose about 17 times on a dry wt. basis and 27 times on a per cell basis that is as much as that of cells grown in 1 ppm 2,4-D. However, the incorporation of phenylalanine-[U-$^{14}$C] into protein was almost the same between both cultures (Table 2). The results were completely contrary to those obtained for the carotenoid formation in the same cell line. In cells grown in 1 ppm 2,4-D, carotenoid was actively accumulated whereas its content decreased after transferring cells into a medium containing 0·1 ppm 2,4-D [5]. These results suggested that 2,4-D may play some regulatory role in the pathway from phenylalanine to cinnamic derivatives or lignin formation. Lignin formation was, in fact, considerably affected by changing the concentration of 2,4-D. The lignin content of cells grown in 0·05 ppm 2,4-D was increased two-fold on the basis of dry weight or per cell compared with that in 1 ppm 2,4-D (Table 5). The activity of phenylalanine ammonia-lyase (PAL) was also affected by the change of auxin concentration. The activity from cells in 0·05 ppm 2,4-D was *ca* 14 times higher than that in 1 ppm 2,4-D after 90 min-reaction. Several workers have already reported that PAL is induced by plant hormone ethylene [7, 8] and also by light [9–11]. The direct correlation between PAL activity and the formation of phenylpropanoid has been established by many workers [12]. Therefore, the marked increase in the content of phenolic acid and lignin by lowering the concentration of 2,4-D might be caused by the increase in the PAL activity.

Table 5. The lignin contents of both cell cultures in 0·05 ppm and 1 ppm 2,4-D

| Concentration of 2,4-D (ppm) | Content | | |
|---|---|---|---|
| | mg/g fr. wt | mg/g dry wt | pg/cell |
| 0·05 | 2·9 | 37·2 | 70 |
| 1·0 | 1·8 | 18·9 | 32 |

## EXPERIMENTAL

*Cultured cells.* The cell line GD-2 used in this expt was derived from a storage root of red carrot cu *Kintoki* and maintained in a liquid medium [13] containing 1 ppm 2,4-D in place of indolylacetic acid and kinetin. The detailed procedure of cell cultivation was described in our previous paper [14].

*Extraction of phenolic acid.* Cultured cells were collected by filtering and washed with $H_2O$. The washed cells were extracted with preboiled 80% EtOH for 1 hr and then 0·5 hr. The combined extract was stored at $-4°$ for 18 hr and filtered through a Buchner funnel. After the filtrate was evaporated to dryness, the residue was dissolved in $H_2O$ and saponified with 2 N NaOH for 2 hr under a stream of $N_2$. The saponified soln was acidified with HCl and extracted with $Et_2O$. After shaking the ether extract with 5% aq. $NaHCO_3$, the bicarbonate fraction was acidified and reextracted with $Et_2O$. The ethereal fraction was evaporated to dryness and the residue was dissolved in an appropriate amount of EtOH.

*Qualitative and quantitative assays of phenolic acids.* Phenolic acids were separated by PC with $C_6H_6$–HOAc–$H_2O$ (6:7:3.) as solvent. The acids were detected under UV with $NH_3$ vapour and identified by their $R_f$ values and color with the sulphanilic reagent [15]. For quantitative assay, areas of phenolic acids detected under UV were cut out and eluted with EtOH. The assay was spectrophotometric [2].

*Incorporation of phenylalanine-[U-$^{14}$C] into phenolic acid and protein.* After L-phenylalanine-[U-$^{14}$C] (3 $\mu$Ci) was added to 150 ml of the cell suspension on each culture day, culture was continued for 6 hr. Phenolic acids were determined quantitatively and their radioactivities were counted in a solution of toluene–EtOH–POPOP with a liquid scintillation counter. In addition, each aliquot of the above cells was ground with quartz sand and centrifuged at 10000 *g* for 15 min. To the supernatant was added TCA (final concn 1·1 M). The ppt was washed with 1·1 M TCA and dissolved in 0·01 M NaOH. The radioactivity was counted as described above and the protein content determined after ref 16.

*Quantitative assay of lignin.* The assay was carried out after the slightly modified procedure described [17]. Cultured cells were twice extracted with EtOH–$C_6H_6$ (1:2) for 2 hr and washed with EtOH and $Et_2O$. The dried cell debris was suspended in 0·5% pepsin in 0·1 M HCl and incubated at 40° for 18 hr. After centrifugation, the ppt was washed with hot $H_2O$ and refluxed with 5% $H_2SO_4$ for 1 hr. The refluxed material was washed with $H_2O$, EtOH, $Et_2O$ and then treated with 72% $H_2SO_4$ for 2 hr at 20°. The crude lignin obtained by adding $H_2O$ was again refluxed with 3% $H_2SO_4$ for 2 hr. The sample was washed, dried at 105°, weighed and ashed. The yield of lignin was calculated on an ash-free basis.

*Assay of PAL.* The extraction of PAL was principally done after ref. 8. Cultured cells were disrupted with quartz sand in 3 vol of 25 mM borate buffer (pH 8·8) containing 5 mM $\beta$-mercaptoethanol and the homogenate was centrifuged at 10000 *g* for 15 min. The clear yellow supernatant was dialysed against the same borate buffer. The dialysed supernatant was referred to as the enzyme preparation. The reaction mixture contained 0·2 ml of the enzyme (500 $\mu$g protein), 100 $\mu$mol of borate and 15 $\mu$mol of L-phenylalanine in a total vol of 3 ml at pH 8·8. The mixture which had no phenylalanine was used as the control. The enzyme activity at 40° was spectrophotometrically measured as the increase in A at 290 nm. The increase in A was found to be proportional to the amount of enzyme.

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