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# Phenylpropanoid Metabolism in Suspension Cultures of *Vanilla planifolia* Andr.[1]

## II. Effects of Precursor Feeding and Metabolic Inhibitors

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### ABSTRACT

**Feeding of cinnamic acid and ferulic acid to non-treated and chitosan-treated cell suspension cultures of *Vanilla planifolia* resulted in the formation of trace amounts of *p*-hydroxy benzoic acid (5.2 micrograms per gram fresh weight of cells) and vanillic acid (6.4 micrograms per gram fresh weight of cells), respectively. Addition of a 4-hydroxycinnamate: CoA-ligase inhibitor, 3,4-(methylenedioxy)-cinnamic acid (MDCA), resulted in a reduced biosynthesis of ligneous material with a simultaneous significant increased vanillic acid formation (around 75 micrograms per gram fresh weight of cells). A $K_i$ of 100 micromolar for 4-hydroxycinnamate: CoA-ligase in a crude preparation was estimated for this inhibitor. It is suggested that the conversion of cinnamic acids into benzoic acids does not involve cinnamoyl CoA esters as intermediates. Feeding of $^{14}$C-cinnamic acid and $^{14}$C-ferulic acid to cells treated with MDCA indicate that cinnamic acid, but not ferulic acid, is a precursor of vanillic acid in these cultivated cells of *V. planifolia*.**

Natural vanillin is a complex flavor obtained from cured *Vanilla* capsules. The main component is vanillin, an aromatic aldehyde. The biosynthesis of this relatively simple compound has not yet been evaluated. It has been suggested that ferulic acid, a central intermediate of the general phenylpropanoid pathway, is converted to vanillin by a reaction analogous to that of the β-oxidation of fatty acids (13).

In the preceding communication we have reported on some studies on cell suspension cultures of *Vanilla planifolia* Andr. (4). This culture was established to investigate the possibility to produce vanillin in culture. However, under all the conditions tested no extractable vanillin could be detected. Elicitation of the cell culture with chitosan resulted in an induction of various enzymes, *e.g.* PAL[4], 4CL, and CAD. An increased synthesis of ligneous material took place in the elicited cells (4). In fact, most of the phenylpropanoids appeared to be rapidly incorporated into such polymeric materials after elicitation. The fact that no vanillin could be extracted from the cultivated cells could be the result of either a lack of enzymes involves in the biosynthesis of this compound or to the channeling of substrates into ligneous material. To evaluate these two possibilities, experiments with precursor feeding and addition of metabolic inhibitors to the cultures of *V. planifolia* have been carried out.

## MATERIALS AND METHODS

### Chemicals

Feruloyl-CoA and OHPAS were kind gifts of Dr. N. Amrhein, ETH, Zürich, Switzerland, and AOPP was kindly supplied by Dr. R. Chollet, Sandoz AG, Basel, Switzerland. MDCA, 4-methoxycinnamic acid and 3,4-dimethoxycinnamic acid were obtained from Aldrich, Steinheim FRG. All other phenolic compounds and β-glucosidase were from Fluka, Buchs, Switzerland. [side chain-3-$^{14}$C]Cinnamic acid (56 mCi/mmol) and [$^{14}$C]methyl iodide (58 mCi/mmol) were supplied by Amersham, Little Chalfont, UK. Chitosan was from Chugai Boyeki Europe, Düsseldorf, FRG, and MS-medium was from Flow Laboratories, Rickmansworth, UK.

### Synthesis of [O-$^{14}$C-Methyl]Ferulic Acid

#### *4-Benzylprotocatechuic Aldehyde*

4-Benzylprotocatechuic aldehyde and vanillin were prepared by a modification of the procedure described by Haider and Lim (8). Protocatechuic aldehyde (25 mmol) was dissolved in methyl ethyl ketone (17 mL) and $K_2CO_3$ (5 g) and benzyl chloride (25 mmol) were added. After refluxing at 90°C for 6 h, the solvent was evaporated. The residue was dissolved in methyl ethyl ketone and extracted three times with NaOH (0.1 M). The aqueous phases were pooled, acidi-

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[4] Abbreviations: PAL, L-phenylalanine ammonia-lyase; AOPP, α-aminooxy-β-phenylpropionic acid; CAD, coniferyl alcohol dehydrogenase; CCR, cinnamoyl-CoA reductase; MDCA, 3,4-methylenedioxy cinnamic acid; OHPAS, *N*-(*O*-aminophenyl)sulfinamoyl-tertiobutyl acetate; 4CL, 4-hydroxycinnamate: CoA ligase.

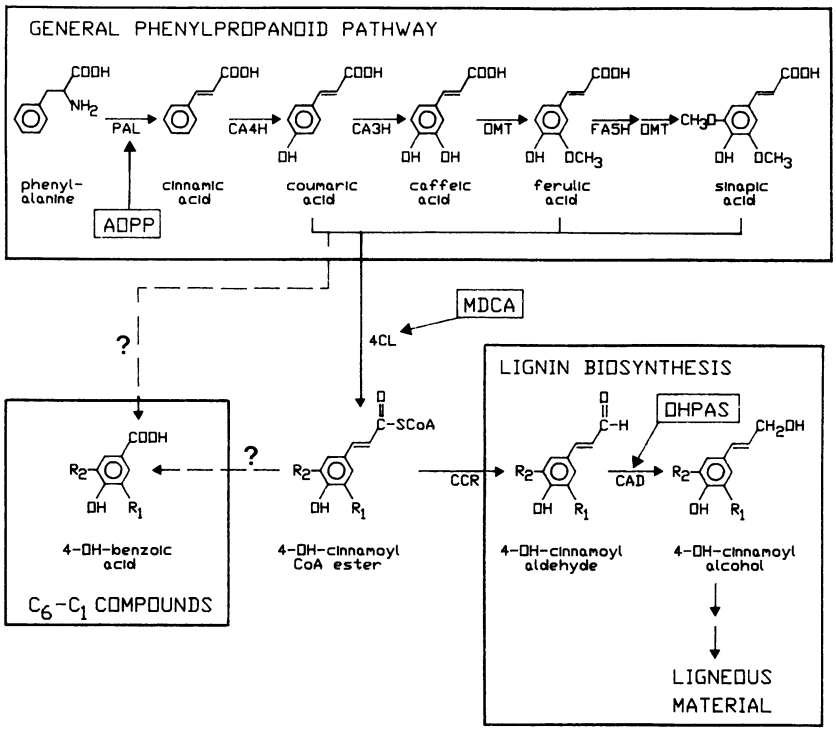



**Figure 1.** Summary of some of the enzymatic reactions of the phenylpropanoid metabolism. Enzymes: PAL, phenylalanine ammonia lyase (EC 4.3.1.5); CA4H, *t*-cinnamic acid 4-hydroxylase (EC 1.14.13.11); CA3H, *p*-coumaric acid 3-hydroxylase (not classified); OMT, catechol *O*-methyltransferase (EC 2.1.1.6); FA5H, ferulic acid 5-hydroxylase (not classified); 4CL, 4-hydroxycinnamate: CoA ligase (EC 6.2.1.12); CCR, cinnamoyl-CoA reductase (EC 1.2.1.44); CAD, cinnamyl alcohol dehydrogenase (EC 1.1.1.-).

fied (6 N HCl [pH 3]) and then extracted three times with methyl ethyl ketone, which subsequently was removed under reduced pressure. The product was purified by crystallization from 60% methanol.

### *[O$^{14}$C-Methyl]Vanillin*

4-Benzyl protocatechuic aldehyde (10 μmol) was dissolved in methyl ethyl ketone (1 mL) and $K_2CO_3$ (20 mg) was added. [$^{14}$C]Methyl iodide (100 μCi; 58 mCi/mmol) was transferred under vacuum into the reaction flask, which was sealed and incubated for 1 h at room temperature and then at 60°C for 16 h. After evaporation of the solvent, the residue was stirred with 6 N HCl (10 ml) at 120°C for 1 h under a constant flow of $N_2$. Vanillin was then extracted with diethyl ether and purified by preparative TLC (yield: 55% from [$^{14}$C]methyl iodide; 150 μg vanillin; 56 mCi/mmol).

### *[O-$^{14}$C-Methyl]Ferulic Acid*

[*O*-$^{14}$C-methyl]Vanillin was converted to [*O*-$^{14}$C-methyl] ferulic acid by a modification of the method described in Kratzl *et al.* (10). [*O*-$^{14}$C-methyl]Vanillin (55 μCi) was diluted with unlabeled vanillin (20 mg) and dissolved in pyridine (100 μL). Aniline (4 μL) and malonic acid (32 mg) were added. The flask was sealed and incubated at 65°C for 7 h. Formed ferulic acid was precipitated after addition of $H_2O$ (3 mL) by addition of 3 N HCl (500 μL). The crystalized product was washed two times with 2 N HCl and dried (yield: 66%, 0.30 mCi/mmol).

### Cell Culture

Suspension cultures of *Vanilla planifolia* Andr., supplied by Danisco Biotechnology Denmark, were cultivated on a gyratory shaker (120 rpm) in the dark at 26°C. MS medium supplemented with sucrose (30 g/L) and NAA (1 mg/L) was used. Cells were routinely subcultured every 10 d by transferring an inoculum of 7% (fresh weight) to fresh medium.

### Elicitation

Elicitation with chitosan was carried out as previously described (4).

### Precursor Feeding

For feeding experiments cell suspensions in the late logarithmic growth phase (*e.g.* d 6–7) were pooled and divided into small flasks. Cinnamic acids were added as sterile-filtered solutions of the sodium salts of the corresponding acids in water.

### Enzyme Extraction and Assay

CCR was extracted from frozen cells (1.0 g) with 100 mM sodium phosphate buffer (pH 7.0) (2.0 mL) in a glass homogenizer. After centrifugation at 10,000*g* for 20 min the supernatant was desalted over a Sephadex G-25 column.

CCR activity in the desalted extracts was determined in a spectrophotometric assay. Crude extract (50 μL) was incu-

bated, in a total volume of 0.5 mL, with 50 μmol sodium phosphate (pH 6.4) and 0.25 μmol NADPH. The reaction was started by addition of 25 nmol of feruloyl-CoA and the decrease in absorbance was recorded at 366 nm. An extinction coefficient of 14.9 $10^3$ was used (12).

Other enzymes were extracted and assayed as previously described (4). Extracts desalted over Sephadex G-25 were used for all enzymatic assays.

### Extraction and Quantification of Soluble Phenolics

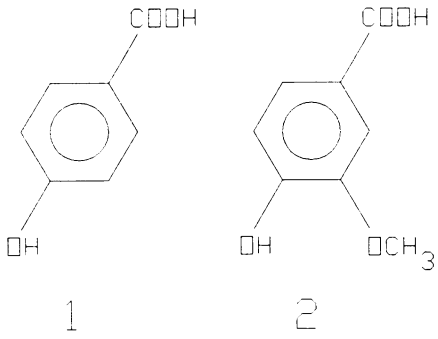
Frozen cells (1 g) were homogenized in 100 mM acetate buffer (pH 5.0) (2 mL). After centrifugation (10,000*g*, 5 min) the supernatant was incubated with β-glucosidase (0.2% final concentration) at 37°C for 3 h. HPLC analysis of the extracts was performed as previously described (4).

## RESULTS AND DISCUSSION

Some reactions of the phenylpropanoid metabolism, which are relevant to the following discussion, are shown in Figure 1.

### Effect of Precursor Feeding on Product Formation

In a first set of experiments two intermediates of the phenylpropanoid pathway, *i.e.* cinnamic acid and ferulic acid (*cf.* Fig. 1), were fed to nontreated and elicited cells of *Vanilla planifolia*. High concentrations (5 mM) of these intermediates were toxic to the cells as judged by viability staining with fluoresceine diacetate. Therefore, the precursors were fed at a moderate concentration (1 mM). The rates of uptake of the fed precursors were essentially the same for nontreated and elicitor-treated cells.

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**Scheme 1**

The results of these feeding experiments are summarized in Figure 2. Ferulic acid and coniferyl alcohol were the only phenylpropanoids that could be detected in extracts of cells grown under standard conditions (Fig. 2A). After elicitation the concentration of these intermediates was considerably lower (Fig. 2B). It has previously been shown that elicitation of the cells by chitosan results in an increased incorporation of phenylpropanoids into ligneous material (4) (*cf.* Fig. 1).

After addition of cinnamic acid to nonelicited cells a number of intermediates of the general phenylpropanoid pathway (*i.e.* 4-coumaric, caffeic, and ferulic acid and coniferyl alcohol) could be seen transiently (Fig. 2C). Traces of 4-hydroxybenzoic acid (5.2 μg per g fresh weight cells) were also detected. The same intermediates could be identified after feeding of cinnamic acid to elicitor-treated cells (Fig. 2D). However, the concentrations of these intermediates were lower indicating that they were more rapidly metabolized in elicited cells.

Addition of ferulic acid to nontreated and elicited cells resulted in the formation of coniferyl alcohol and traces of

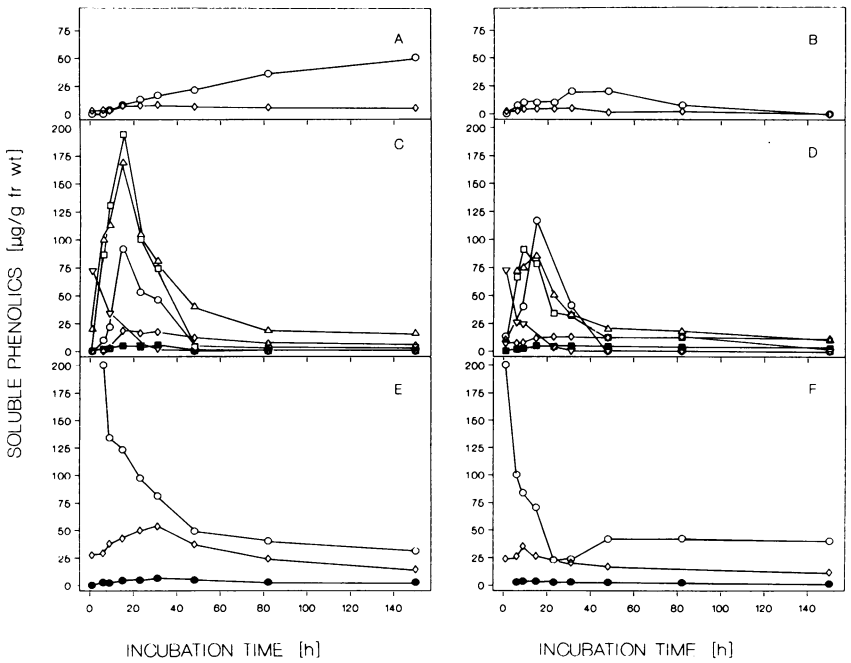


**Figure 2.** Time course of product accumulation in nontreated (A, C, and E) and chitosan-treated (B, D, and F) suspension cultures of *V. planifolia* without precursor feeding (A and B) or with addition of 1 mM cinnamic acid (C and D) or 1 mM ferulic acid (E and F). Feeding and induction with chitosan (830 μg/g fresh weight) were carried out with 6 d old suspension cultures. The samples were treated with β-glucosidase as described in "Materials and Methods.' (▽), Cinnamic acid; (□), 4-coumaric acid; (△), caffeic acid; (○), ferulic acid; (◇), coniferyl alcohol; (■), 4-hydroxybenzoic acid; (●), vanillic acid.

**Table I.** *Apparent Inhibition Constants of Various 4-Methoxy Cinnamic Acids Toward 4CL from Vanilla planifolia*

Ferulic acid was used as the variable substrate. The concentrations of ATP and CoA in the assay were 1.0 mM and 100 μM, respectively.

| Inhibitor | $K_i$ |
|---|---|
| | μM |
| 4-Methoxy cinnamic acid | 300 |
| 3,4-Dimethoxy cinnamic acid | 500 |
| 3,4,Methylenedioxy cinnamic acid | 100 |

vanillic acid (6.4 μg per g fresh weight of cells) (Fig. 2, E and F).

The fact that traces of 4-hydroxybenzoic (1) and vanillic acid (2) (Scheme 1) could be detected by HPLC after feeding cinnamic and ferulic acid, respectively, indicates the presence of an enzymatic system capable of converting $C_6$-$C_3$ compounds (*i.e.* hydroxycinnamic acids) into $C_6$-$C_1$ compounds (*i.e.* hydroxybenzoic acids) within the cells (*cf.* Fig. 1). However, the concentrations of these $C_6$-$C_1$ substances are very low indicating that the fed precursors are mainly channelled into other metabolic pathways (*e.g.* biosynthesis of ligneous materials). Inhibition of such competing pathways may lead to the formation of increased amounts of benzoic acid derivatives.

### Metabolic Inhibitors of the Phenylpropanoid Metabolism

Figure 1 shows the general phenylpropanoid pathway operating in cells of *V. planifolia* leading from phenylalanine to ligneous material. The biosynthetic route from cinnamic acids to benzoic acids is not known. Two alternative pathways have been included in Figure 1. A major question is whether the conversion of $C_6$-$C_3$ compounds to $C_6$-$C_1$ compounds in higher plants involves cinnamoyl CoA esters or not.

In fact, a number of studies on the formation of 4-hydroxybenzoic acids in higher plants have indicated that the reaction from cinnamic acids to the corresponding benzoic acids does not proceed via cinnamoyl-CoA esters (1, 3, 7, 11). In these studies no requirement of the reaction for added CoA could be proven.

A key enzyme in the biosynthesis of ligneous materials is 4CL catalyzing the formation of hydroxycinnamic acid CoA esters (Fig. 1). Inhibition of this enzyme under *in vivo* conditions could give information on the involvement of cinnamic acid CoA esters in biosynthesis of benzoic acids.

We have tested various 4-methoxylated cinnamic acids for their inhibitory effect on 4CL. These substances were selected as potential inhibitors of this enzyme, since it has been shown that they are very poor substrates for this enzyme from *Forsythia suspensa* (14) and *Petroselinum hortense* (9).

*In vitro* studies were carried out with a crude enzyme preparation from cells of *V. planifolia*. From Lineweaver-Burke plots a $K_m$ value of 8.4 μM for ferulic acid may be estimated for this crude 4CL preparation. A $K_m$ of 33 μM for ferulic acid was previously determined for a partially purified 4CL from cultivated parsley cells (9). Furthermore, it may be concluded from Lineweaver-Burke plots that MDCA is a competitive inhibitor. An apparent $K_i$ value of 100 μM MDCA has been calculated from a plot of the slopes of lines in Lineweaver-Burke plots against inhibitor concentration used. Three different concentrations of the inhibitor were used for these determinations.

The estimated $K_i$ values for the three inhibitors tested are summarized in Table I. It is evident that MDCA is the best inhibitor of 4CL in crude cell-free extracts. The effects of this inhibitor on various other enzymes of the phenylpropanoid pathway was investigated with cell free protein extracts. The activity of PAL, OMT, and CAD (*cf.* Fig. 1) was essentially unaffected, while a noncompetitive inhibition of CCR with respect to ferulic acid as substrate could be established. How-

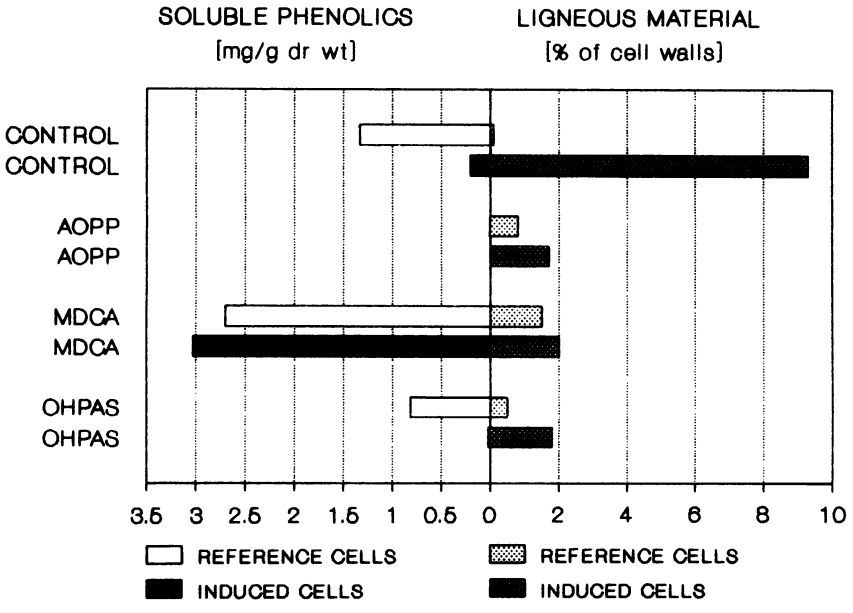


**Figure 3.** Effect of various inhibitors on the phenylpropanoid metabolism in suspension cultures of *V. planifolia*. Inhibitors were fed to 7-d old suspension cultures at a concentration of 1 mM. Cells were extracted after an incubation period of 150 h. Induced cells were treated with 740 μg chitosan per g fresh weight of cells.

**Table II.** *PAL and 4CL Activity in Cells of V. planifolia after Various Treatments*

The cells were extracted 24 h after treatment.

| Treatment | Specific Enzyme Activity | |
|---|---|---|
| | PAL | 4CL |
| | *nmol/min/mg protein* | |
| None | 0.25 | 0.85 |
| Chitosan (400 μg/mL) | 2.70 | 4.20 |
| MDCA (500 μM) | 1.38 | 3.30 |

ever, the inhibition constant for this enzyme is two times higher than that for 4CL. It may therefore be assumed that under *in vivo* conditions 4CL is inhibited to a considerably larger extent than CCR.

In addition to MDCA two other metabolic inhibitors, AOPP inhibiting PAL (2) and OHPAS inhibiting CAD (6), were also used in the subsequent *in vivo* studies (*cf.* Fig. 1).

### Effects of Metabolic Inhibitors on Phenylpropanoid Metabolism

The three selected inhibitors, *i.e.* AOPP, OHPAS, and MDCA, were added to nontreated and chitosan-treated cell cultures of *V. planifolia*. The effects of these inhibitors on phenylpropanoid metabolism are summarized in Figure 3.

Cells grown under standard conditions produced some extractable phenolics while elicitor-treated cells synthesized a significant amount of ligneous material (almost 10% of the isolated cell walls). Essentially, no soluble phenolics could be extracted from elicited cells.

When AOPP was added to nontreated and elicited cell cultures no soluble phenolics could be detected. This is in accordance with the fact that AOPP is an effective inhibitor of PAL, the entry point enzyme of the phenylpropanoid pathway (*cf.* Fig. 1) (2). No cinnamic acid is produced and therefore the phenylpropanoid pathway is shut off. Due to the lack of phenylpropanoids extensive synthesis of ligneous material is prevented in elicited cells treated with AOPP.

Addition of MDCA to cultures appears to induce the formation of soluble phenolics with a simultaneous inhibition of ligenous material formation. Therefore, considerably more soluble phenolics are extractable from these cells than from cells grown under standard conditions. In fact, this compound induces enzymes of the phenylpropanoid metabolism in a similar manner as chitosan as exemplified in Table II.

MDCA also efficiently inhibits the formation of ligneous material in elicitor-treated cells. This is, to our knowledge, the first example of the utilization of MDCA as a metabolic inhibitor acting on 4CL. These results were encouraging and we have therefore carried out more extensive studies with this inhibitor (see below).

The formation of ligneous material is also inhibited in cells treated with OHPAS. This inhibitor has been reported to specifically inhibit CAD, an enzyme involved in the synthesis of lignin precursors (6) (*cf.* Fig. 1). Consequently, only a branch of the phenylpropanoid metabolism is influenced by this inhibitor and therefore soluble phenolics should accumulate in OHPAS-treated cells. However, the results obtained with elicitor-treated cells indicate that OHPAS must inhibit other early enzyme(s) of phenylpropanoid metabolism. Even though the formation of ligneous materials is inhibited in these elicited cells, no accumulation of soluble phenolics is observed. The elicitor-induced PAL should produce soluble phenolics in a manner similar to that observed in cells treated with MDCA. Therefore, the results obtained may be explained by an inhibition of PAL by OHPAS (*cf.* AOPP). In fact, preliminary experiments have shown that PAL is inhibited by relatively low concentrations of OHPAS and consequently this inhibitor appears not to be specific for CAD. Further studies are in progress in our laboratory to evaluate this observation further.

### Effect of MDCA on Soluble Phenolics Formation

Addition of MDCA (1 mM) to a culture of *V. planifolia* under normal growth conditions resulted in the formation of soluble phenolics (Fig. 3) in particular of significant amounts of vanillic acid as shown in Figure 4. Vanillic acid was isolated from cell free extracts by preparative TLC. The isolated compound was confirmed to be vanillic acid by its UV spectrum and its comigration with authentic vanillic acid in two different TLC systems (silica and cellulose) and in the HPLC system.

The inhibitor itself appeared not to be metabolized by the cells (*cf.* Fig. 4). This was also confirmed by a quantitative determination of MDCA and vanillic acid in cell extracts. After addition of 4.2 μmol MDCA per flask (10 mL cell suspension) 4.0 μmol MDCA and 1.0 μmol vanillic acid could be isolated after 100 h incubation (data not shown).

Accumulation of vanillic acid started when the concentration of MDCA within the cells had reached a high steady state level (15–20 h after addition of MDCA). The apparent inhibition of 4CL involved in the synthesis of lignin precursors under *in vivo* conditions resulted in an increased formation

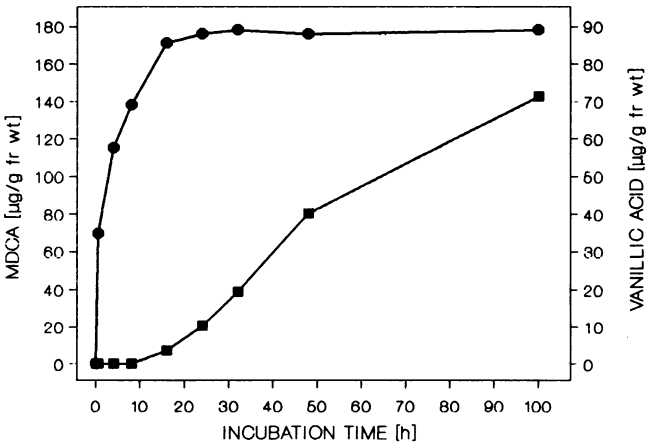


**Figure 4.** Time course of vanillic acid formation in *V. planifolia* cells in the presence of MDCA. MDCA (1 mM) was fed to a 6-d old suspension culture. The samples were treated with β-glucosidase as described in "Materials and Methods." (■), Vanillic acid; (●), MDCA.

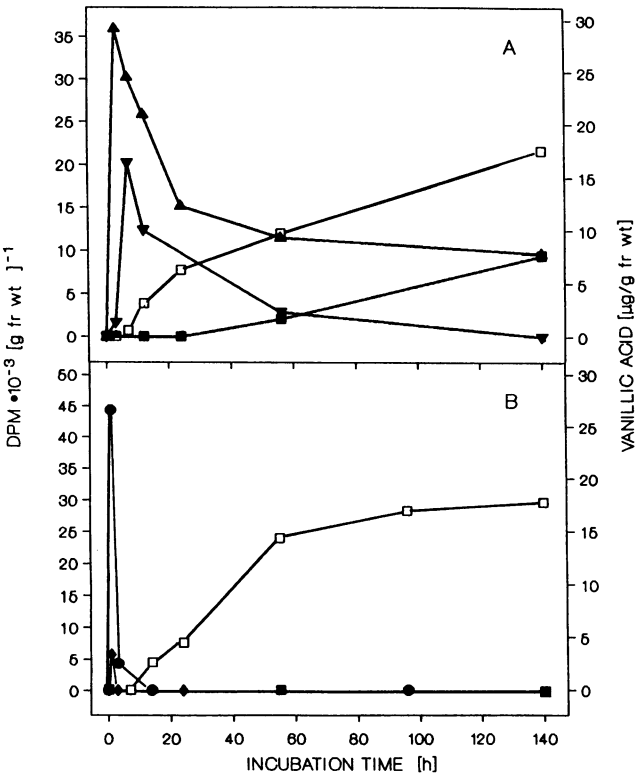


**Figure 5.** Time course of intracellular metabolite concentrations in cell cultures of *V. planifolia* in the presence of MDCA (1 mM) after feeding $^{14}$C-cinnamic acid (A) or $^{14}$C-ferulic acid (B). Cinnamic and ferulic acid were fed at concentrations of 30 and 320 µM, corresponding to 0.15 and 0.68 µCi per g fresh weight of cells, respectively. Extracts were treated with β-glucosidase as described in "Materials and Methods." A: (▲), [$^{14}$C]Cinnamic acid; (▼), [$^{14}$C]veratric acid; (■), [$^{14}$C]vanillic acid; (□), total vanillic acid. B: (●), [$^{14}$C]Ferulic acid; (◆), $^{14}$C-labeled unidentified intermediate; (■), [$^{14}$C]vanillic acid; (□), total vanillic acid.

of $C_6$-$C_1$ compounds. These results indicate that feruloyl-CoA is unlikely to be an intermediate in vanillic acid biosynthesis in these cultivated cells (*cf* Fig. 1). Furthermore, it may be concluded from our experiments (Figs. 3 and 4) that MDCA acts as a metabolic inhibitor under *in vivo* conditions.

Feeding experiments with precursors (cinnamic and ferulic acid) in the presence of the inhibitor (MDCA) were subsequently carried out. For these experiments $^{14}$C-labeled precursors were used in order to follow the incorporation of the fed precursors into other metabolites. The results of these experiments are summarized in Figure 5.

After 140 h incubation around 3% of the fed[$^{14}$C]cinnamic acid had been incorporated into vanillic acid (Fig. 5A). At this stage no $^{14}$C-labeled vanillic acid could be isolated from cultures fed with [$^{14}$C]ferulic acid (Fig. 5B). However, significant amounts of nonlabeled vanillic acid could be extracted from these cells (open symbols in Fig. 5B). These results indicate that cinnamic acid but not ferulic acid is a precursor of vanillic acid synthesis in this cell culture. Feeding experiments carried out with vanilla capsule tissue, however, have shown that [$^{14}$C]ferulic acid is efficiently incorporated into vanillin (13). Apparently, the biosynthetic capacity of undifferentiated cultivated cells differ from that of natural capsule tissue under the conditions used. Further studies are in progress in our laboratory to evaluate this discrepancy and to investigate the biosynthetic pathway converting $C_6$-$C_3$- to $C_6$-$C_1$-compounds (*e.g.* 4-hydroxybenzoic and vanillic acid) in the suspension culture of *V. planifolia* (5).

## CONCLUSION

Formation of vanillic acid in cell cultures of *Vanilla planifolia* occurs after addition of a 4CL inhibitor (MDCA). MDCA appears to be a potent metabolic inhibitor acting on 4CL and therefore these observations suggest that the formation of benzoic acid derivatives in the culture is dependent on an enzyme system not involving 4CL. Furthermore, cinnamic acid, but not ferulic acid, is a precursor of vanillic acid in cells treated with MDCA. Consequently, the branching point of the pathway leading to vanillic acid has to be located before ferulic acid in the general phenylpropanoid pathway.

## ACKNOWLEDGMENT

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