Interference by a Phenylacetate Pathway in Isotopic Assays for Phenylalanine Ammonia-Lyase in Leaf Extracts

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ABSTRACT

Particulate and soluble fractions from leaves of Sorghum, Spinacia (spinach), and Coleus, capable of metabolizing l-phenylalanine to cinnamate or to caffeate, are also able to convert l- and d-phenylalanine to phenylacetate. Since cinnamate and phenylacetate are not effectively separated in commonly used chromatographic solvents, some of the isotopic assays used for phenylalanine-ammonia-lyase are rendered ambiguous by the interference of this second pathway. Therefore, a "double decker," two-dimensional paper chromatographic method was designed to separate cinnamate and phenylacetate. This was combined with the use of phenylalanine labeled randomly or just in either the carbon 1 or 2 position of the side chain.

During attempts to demonstrate the presence of a particulate complex in leaves capable of producing caffeate from l-[U-14C]phenylalanine, it became apparent that a presumed cinnamate area isolated by paper chromatography using organic solvents such as benzene contained another compound. The major contaminant has been identified as the Ce-C2 compound phenylacetate. Since its presence would interfere with commonly used isotopic assays for cinnamate involving toluene extraction of the products of an incubation mixture or isolation of cinnamate chromatographically using organic solvents prior to scintillation counting, methods were designed to separate phenylacetate from cinnamate and to compare the two pathways quantitatively using two enzyme assay methods. One was the frequently used method for measuring PAL activity; the other was designed to detect multiple steps in the Ce-C2 pathway from phenylalanine to caffeate in particulate preparations with as many intact organelles and vesicles as possible. Light was used during the incubation in the Sequence assay because of the possibility of the importance of chloroplasts in phenolic metabolism.

MATERIALS AND METHODS

Preparation of Leaf Extracts. About 6- to 7-day-old Sorghum bicolor plants were grown in the light at 30 C as in previous studies (9, 10). Commercially purchased spinach (Spinacia oleracea) leaves were kept in moist trays under cool white fluorescent lamps (2.100 lux) at 25 C for 20 to 24 hr prior to extraction. Greenhouse-grown Coleus blumei plants, an unknown cultivar with dark red leaves edged with green, were transferred to the growth chamber used for sorghum for about 48 hr prior to extraction. Two basic extraction mixtures were used. Both contained 100 mM Tricine buffer (pH 8), 0.6 M sorbitol, 3 mM Ca(NO3)2, and 2% (w/v) soluble PVP-40 (Sigma Chemical Co.). One contained 10 mM ME, the other 0.1% (w/v) ascorbate. About 10 g fresh wt of leaves were ground in 40 ml of medium. After removal of residues trapped by cheesecloth and centrifugation at 500g, the pellet obtained at 37,000g (P) was resuspended in 2 ml of 0.6 M sorbitol, 3 mM Ca(NO3)2, in 100 mM Tricine (pH 8) for the Sequence assay, or in 50 mM borate (pH 8.4) for the PAL assay. The supernatant fraction at 37,000g was precipitated with 500 g/l of ammonium sulfate. A pellicle at the surface was formed instead of a pellet, apparently due to the use of soluble PVP. This surface layer was resuspended in the appropriate medium for the PAL or Sequence assay. Separate extracts were made for each type of assay.

PAL Assay. A 1-ml mixture in 50 mM borate buffer (pH 8.8) contained 4 mM L-phenylalanine, 0.25 μCi of l-[U-14C]phenylalanine (0.6 μM), or 0.50 μCi of dl-[2-14C]phenylalanine (20 μM), or 0.25 μCi of l-[1-14C]phenylalanine (4 μM), purchased from either New England Nuclear or American-Searle, 1 mM ME when noted, enzyme extract equivalent to 0.25 or 0.5 g fresh wt of tissue for pellet fractions, and 0.1 fresh wt equivalent for the supernatant fraction. The tubes were incubated in laboratory light for 2 hr at 30 C with shaking.

Sequence Assay. A 1-ml mixture in 100 mM Tricine (pH 8) and 0.3 M sorbitol contained 4 mM L-phenylalanine and 14C compounds as in the PAL assay, 20 mM ascorbate, 1.5 mM Ca(NO3)2, 0.5 mg of BSA, 5 mM ME when noted, plus enzyme extracts as in the PAL assay. The tubes were incubated under fluorescent lights (1,000 lux) for 3 hr at 30 C with stirring.

Analysis of Incubation Mixtures. After the addition of 30 μl of 1 N HCl, the incubation mixture was analyzed either by removing a 0.1-ml aliquot for paper chromatography on Whatman No. 1 paper (23 × 45 cm) as described in the text, or the entire incubation medium was extracted first with petroleum ether (5 × 1-ml aliquots) and then with ethyl acetate (3 × 1 ml). Addition of methanol to about 30% (v/v) was necessary to facilitate removal of the Chl with the petroleum ether extraction. Both of these extracts were concentrated and applied to chromatograms. Standards of cinnamic (10 μg), phenylactic (300 μg), caffeic acids (2 μg), and other selected compounds were added to the chromatograms and were identified under UV. Other radioactive areas were identified by detection with a Geiger counter or by x-ray film autoradiography. Identified spots were cut out and added to a PPO-POPPO paleuene medium for scintillation counting (9). Quench channel ratios were used to determine efficiencies. Activity was reported as dpm/hr/g fresh wt, which can be converted to nmol/hr/g fresh wt by multiplying by 7.3 × 10-3. Specific radioactivities were determined experimentally.

Chromatographic solvents used were: BeAW: benzene-acetic acid-water (40:15:0.7); BuNW: butanol-ammonium hydroxide-water (40:5:5); BAW: butanol-acetic acid-water (30:5:10) (all w/v), 50 mM phosphate buffer (pH 7).

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2 Abbreviations used: PAL: phenylalanine ammonia-lyase; ME: 2-mercaptoethanol.

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RESULTS

Separation of Cinnamate and Phenylacetate by Paper Chromatography. Neither paper nor silica gel TLC with various mixtures containing organic solvents was capable of separating phenylacetic and cinnamic acids. Effective separation was found using 50 mM phosphate buffer (pH 7) combined with two other solvent mixtures in double decker, two-dimensional paper chromatography.

Aliquots (0.1 ml) of the acidified incubation mixture were either directly chromatographed (short method) or both a petroleum ether extract and a subsequent ethyl acetate extract of the entire incubation medium were chromatographed separately (long method). The papers were chromatographed first in the long dimension with BeAW until the solvent front migrated halfway up the length of the chromatogram (Fig. 1). After drying, the paper was cut about 1 cm below the cinnamate-phenylacetate area. This top portion was then chromatographed in the same direction in 50 mM phosphate buffer (pH 7), and subsequently in the second dimension in BuNW. This third solvent was not necessary in some sorghum preparations but was required in the case of spinach mixtures using the short method. Three solvents were always used with the long method. The middle and bottom portions were developed in the second dimension in BuNW and BAW, respectively, for the best separations.

While this method effectively separates trans-cinnamate and phenylacetate, the latter area overlaps with that of cis-cinnamate, benzoate, and phenylpyruvate, all possible products of phenylalanine. Estimates of the degree of contamination of this phenylacetate area with these other compounds were made by various methods, including x-ray autoradiography, scintillation counting of areas bordering the phenylacetate area, differential solubility in petroleum ether, and the use in the incubation mixture of phenylalanine labeled in various portions of the C₆ side chain.

Extent of Interference in Various Preparations. Using L-[U-¹⁴C]phenylalanine in either the PAL or Sequence assays, the incorporation of label into compounds that migrate together to the solvent front in BeAW, but which can be separated subsequently by further chromatography in a phosphate solvent into trans-cinnamate and phenylacetate complex areas, are shown in Table 1. Generally, very little of the label in the latter area was soluble in petroleum ether, indicating that neither cis-cinnamate nor benzoate was a major product. In general, sorghum preparations showed greater activity for PAL than for the phenylacetate acid pathway, while just the opposite was true for spinach. Coleus preparations were approximately intermediate. It should be remembered that in either assay, the label accumulated at any one time might actually be less than the amount synthesized if either cinnamate or phenylacetate is further metabolized.

The reactions were linear for about 3 hr. Boiling eliminated the incorporation into both products. There was no evidence of significant nonenzymic products with incubation mixtures minus enzyme. Furthermore, a linear enzyme concentration curve could be extrapolated to zero product. While a linear enzyme concentration curve could be obtained, the range was very narrow, especially in the case of the phenylacetate acid pathway. The cause of this nonlinearity at higher concentrations of enzyme has not been studied.

Since microorganisms produce many of the compounds studied here, comparisons were made between leaves briefly washed in water only and leaves thoroughly washed with a surfactant-reagent (Aerosol) after a brief surface sterilization of the leaves with 70% ethanol. Furthermore, the supernatant fraction (S₅₀) was filtered through a Millipore filter (0.45-µm pore) prior to use. No significant differences in activity were detected in preparations from leaves receiving these different treatments. Likewise, no exponential increase of the products was observed during time course experiments.

Table 1: The conversion of L-phenylalanine to trans-cinnamate and to compounds in the phenylacetate area, using L-[U-¹⁴C]phenylalanine in both the PAL and Sequence assays.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Chlorophyll</th>
<th>Cinnamate</th>
<th>Phenylacetate area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sorghum leaves</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P (37,000 g pellet)</td>
<td>5.1</td>
<td>0.32</td>
<td>351</td>
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<tr>
<td>PAL assay</td>
<td>54</td>
<td>152</td>
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</tr>
<tr>
<td>Sequence assay</td>
<td>159</td>
<td>348</td>
<td></td>
</tr>
<tr>
<td>SAS</td>
<td>170</td>
<td>14,652</td>
<td>3,454</td>
</tr>
<tr>
<td>PAL assay</td>
<td>9,381</td>
<td>2,317</td>
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</tr>
<tr>
<td>Sequence assay</td>
<td>8,961</td>
<td>2,518</td>
<td></td>
</tr>
<tr>
<td>Spinach leaves</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P (37,000 g pellet)</td>
<td>7.6</td>
<td>0.24</td>
<td>38</td>
</tr>
<tr>
<td>PAL assay</td>
<td>38</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>Sequence assay</td>
<td>54</td>
<td>2,626</td>
<td></td>
</tr>
<tr>
<td>SAS</td>
<td>180</td>
<td>419</td>
<td>2,298</td>
</tr>
<tr>
<td>PAL assay</td>
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<tr>
<td>Colocasia leaves</td>
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<tr>
<td>P (37,000 g pellet)</td>
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<td>PAL assay</td>
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</tr>
<tr>
<td>SAS</td>
<td>250</td>
<td>2,544</td>
<td>1,881</td>
</tr>
</tbody>
</table>

1 PAL assay: 80 mM HE in extraction medium, plus 1 mM HCl in incubation mixture.
2 PAL assay: 80 mM HE in extraction medium, plus 1 mM HCl in incubation mixture.
3 PAL assay: 0.1% ascorbic acid in extraction medium; plus 5 mM HE and 1 mM HCl in incubation mixture.
Effect of Light and Presence of ME. PAL activity in sorghum leaf preparations, both particulate and supernatant fractions, was almost completely dependent upon a light pretreatment of the plant prior to extraction (Table II). Plants grown in the regular 16-hr light/8-hr dark photoperiod, but extracted immediately upon removal from the chamber at the end of the dark cycle, had only 5 to 10% of the activity found in extracts made after the plants had been in the high intensity light for 2 to 4 hr. The specific light requirements have not been studied in further detail. The phenylacetate acid pathway appeared to be relatively light-independent.

ME is frequently added to plant enzyme extracts and to assay protocols for maximum PAL activity. Its effects are complex and differ for sorghum and spinach preparations. The addition of 10 mM ME to the grinding medium was essential for high PAL activity in sorghum as measured by the incorporation of label into cinnamate in the PAL assay (Table II). Its presence during the extraction was less important for the phenylacetate pathway. For sorghum the presence of ME in the incubation mixture produced a consistent, very small inhibition of PAL activity, but drastically reduced the incorporation into phenylacetate. It is possible that the loss of incorporation into the phenylacetate pathway is only apparent, since it might have been converted to other products in the presence of ME. There was some preliminary paper chromatographic evidence of other C_6-C_9 products in the vicinity of o-hydroxyphenylacetate in the presence of ME in the incubation medium. In spinach preparations, the addition of ME to the grinding medium had little effect, but its presence in the assay medium was sometimes beneficial for incorporation of label into both the cinnamate and phenylacetate areas. Therefore, except when noted otherwise, 5 mM ME was present during sequence assays using spinach, but was omitted with sorghum (see Table I). Since the latter preparations were ground with 10 mM ME, some ME that was trapped in the pellets during preparation of the enzyme was always present.

Differential Labeling of C_6-C_9, C_9-C_10, and C_9-C_10 Phenylalanine. Incubation mixtures for both the Sequence and PAL assays were compared using phenylalanine labeled randomly [U-¹⁴C], in only the carbon 2 position of the side chain [2-¹⁴C] or only in the carboxyl group [1-¹⁴C]. Typical data are shown in Table III, corrected for similar specific activities, if it is assumed that only the L form was active even when the D isomer was present. Corrected values in parentheses will be discussed later. The levels of radioactivity incorporated into either the cinnamate or phenylacetate areas were high enough to permitting the use of the short method, but preferably with three solvents, while the low amounts found in the caffeate area required the long or total extraction method. Good agreement was generally found with duplicates done with the short method and with the single determinations analyzed by the long method. Each experiment was repeated at least once.

If only C_6-C_9 products are labeled, all three labeled forms of phenylalanine should give similar results. If differences are found, the incorporation using [2-¹⁴C] subtracted from that with [U-¹⁴C] should give an estimate of the label in C_6-C_9 compounds, while the label in [1-¹⁴C] subtracted from [2-¹⁴C] should give the incorporation into C_9-C_10 compounds. Corrections for the loss of one and two carbons in the case of the [U-¹⁴C] data have not been made. The only C_6-C_9 compound identified so far is phenylacetate; but this area of the chromatogram may be contaminated with benzoate (C_6-C_9) as well as with phenylpyruvate and cis-cinnamate (both C_9-C_10 compounds). The data of Table III show that all of the radioactivity in either the trans-cinnamate or the caffeate areas was due only to C_6-C_9 compounds, since the amount of label incorporated using all three types of labeled phenylalanine was similar.

Estimates of the amounts of benzoate and phenylacetate in this complex area can be made without further correction only in the case of the PAL assay with particulate preparations from sorghum leaves. If it is assumed that the D isomer present in the [2-¹⁴C]substrate is inactive, then the differences in incorporation of label into the phenylacetate region between DL-[2-¹⁴C]phenylalanine and L-[U-¹⁴C]phenylalanine indicate that only small amounts, if any, of benzoate were formed. Also, failure to incorporate label into the phenylacetate region from L-[1-¹⁴C]phenylalanine shows the absence of C_6-C_9 product in this region. Thus, it appears that only phenylacetate is present in this zone. Similar estimates of the amounts of benzoate and phenylacetate in the Sequence assays cannot be made because two to three times the amount of label were found in this area with the [2-¹⁴C] substrate in contrast with the [U-¹⁴C] form. Purification of both radioactive substrates chromatographically did not prevent this unexpected discrepancy.

Effect of D Isomer in Phenylacetate Pathway. Since the [2-¹⁴C] substrate contained the D as well as the L isomer, an experiment was designed to check whether the above discrepancy was due to the presence of an enzyme capable of using the D isomer of phenylalanine (Table IV). When the DL-[2-¹⁴C]phenylalanine was diluted with either nonradioactive L isomer (tube 2) as in the regular Sequence assay or with the D isomer (tube 3), the incorporation of label into the phenylacetate area was similar, indicating that both the D and L forms were being metabolized equally. If this was due to one enzyme, a Dl-decarboxylase (11), the incorporation in tubes 2 and 3 should be twice that of tube 1 with the [U-¹⁴C], since 0.25 μCi of each of the isomers was present. Therefore, the data obtained with the [2-¹⁴C] substrate were divided by two. These corrected
values are shown in parentheses in Tables III and IV. The corrected values for the Sequence assay were sometimes greater than the [U-14C] value. The cause of this has not yet been determined.

Since the corrected values for sorghum preparations in the Sequence assay were equivalent to or higher than those with the [U-14C] substrate (Table III), benzoate was either not formed or small amounts were masked by the extra activity with the D isomer. Spinach preparations, on the other hand, generally gave corrected values for the [2-14C] substrate that were somewhat less than the [U-14C] values, which indicated that the benzoate pathway might be involved. In both cases, however, the major chain-shortening product was the C₅C₃ compound, presumably phenylacetate.

The [2-14C] values in the PAL assay have not been corrected in Table III because there was no evidence of a "doubling" effect due to the presence of the D isomer. Therefore, the activity producing phenylacetate in the PAL assay appears to be due to an enzyme capable of using only the L isomer of phenylalanine. Further corroboration of this hypothesis was found when experiments similar to those described in Table IV for the Sequence assay were done using the PAL assay. The pattern of incorporation into phenylacetate was similar to that into cinnaamate, the latter being produced by PAL enzyme known to be specific for the L isomer and to be inhibited by the D isomer (3) (data not shown).

**Incorporation into Phenylpyruvate.** Significant amounts of dpm in the phenylacetate area with [1-14C] substrate were found consistently only in the case of sorghum particulates in the Sequence assay. Values were generally 30 to 50% of those found with the [U-14C] substrate. The only expected C₅C₃ compounds in this general area are cis-cinnamate and phenylpyruvate. Since the petroleum ether extracts contained relatively little of this label, and cis-cinnamate is much more soluble in the solvent than phenylpyruvate, we tentatively conclude that most of this label is due to phenylpyruvate.

**Other Labeled Compounds.** The radioactive label is widely distributed on the chromatograms from these crude particulate enzyme mixtures. Besides cinnaamate, phenylacetate, caffeate, and possibly benzoate label has been detected in phenyltyrosine and in some unidentified compounds near o-hydroxyphenylacetate and 4-hydroxycinnamate. The latter area is quite complex and has not been studied in detail.

**DISCUSSION AND CONCLUSIONS**

The data presented indicate that significant interference can occur in the PAL or the Sequence assays with [U-14C]phenylalanine if the products of the reaction are isolated with organic solvents such as benzene or toluene. The interference is either increased or decreased in the presence of ME in the incubation medium, depending on the tissue used. While the major interference product appears to be phenylacetate, and in some cases phenylpyruvate, the possibility of benzoate must also be considered (4).

The combination of the double decker chromatographic method and the use of isotopes labeled in different portions of the C₅ side chain adequately separate trans-cinnamate from the major contaminant, phenylacetate, and other C₅-C₃ compounds that would interfere even if [1-14C]phenylalanine were used. However, the chromatographic method alone does not effectively separate phenylacetate, phenylpyruvate, and benzoate. Since phenylpyruvate might be an intermediate in the production of phenylacetate, the use of [1-14C]phenylalanine alone will not protect against interfering compounds unless the double decker paper chromatographic method is used. Significant amounts of presumed phenylpyruvate were accumulated in some cases in the Sequence assay. Erez has already warned about the interference of phenylpyruvate in the spectrophotometric assay for PAL (2). While he attributed its formation to a transaminase, the activity of an amino acid oxidase should also be considered (12).

The interference by this chain-shortening pathway to phenylacetate was greater in the Sequence assay if the D isomer of phenylalanine was present. There appeared to be two types of effects. One, the doubling effect, could be due to an amino acid decarboxylase that functions equally with both isomers, as in the case of 3,4-dihydroxyphenylalanine decarboxylase (11). The cause of the smaller and more variable increase above this doubling effect is unknown.

Phenylacetate production in PAL or multistep assays was not reported by either Albert's (1) or Kindl's group (4) in their studies of similar systems, although both demonstrated the presence of the benzoate pathway. Although the amount of caffeate formed by the *Quercus* particulates was similar to our data, the maximum amount of cinnaamate accumulated in Albert's data was greater than ours unless we would include the phenylacetate value (1). It is not clear whether their separation methods would differentiate between cinnaamate and phenylacetate. The gas chromatographic methods used by Kindl (4), however, distinguished among cinnaamate, benzoate, and phenylacetate.

Since phenylacetate has auxin-like properties and is found in many plants (6), a study of this pathway is of importance in itself, rather than just as an interfering set of reactions in PAL assays. In tissue level studies, Wightman has demonstrated that phenylacetate was produced from phenylalanine mainly by a phenylpyruvate route to phenylacetaldehyde, but that a phenylpyruvate pathway to phenylacetaldehyde was also involved (7). He mentioned the demonstration of the production of phenylacetate in cell-free preparations, but no detail was given (13). Other possible routes include the catalysis by an amino acid oxidase producing phenylpyruvate (12) or a peroxidase forming an amide (5). More than one of these pathways are probably involved in the data reported here. Our tentative conclusion for the crude particulate preparations from sorghum is that a D₆-decarboxylase is active in the Sequence assay, while an L isomer-dependent pathway to phenylacetate predominates in the PAL assay. Benzoate, if present, is considered to be a minor product. Experiments are in progress to characterize the initial reactions in these phenylacetate pathways in order to test this hypothesis. Since no exogenous cofactors were added in these assays, except for ascorbate in the Sequence assay, a knowledge of cofactor requirements will be of prime importance.

**LITERATURE CITED**

