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TYROSINE AMINOTRANSFERASE: THE ENTRYPOINT ENZYME OF THE TYROSINE-DERIVED PATHWAY IN ROSMARINIC ACID BIOSYNTHESIS

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Key Word Index—*Anchusa officinalis*; Boraginaceae; *Coleus blumei*; Lamiaceae; cell cultures; biosynthesis; cell-free system; caffeoyl ester; rosmarinic acid; tyrosine aminotransferase.

Abstract—High activity of tyrosine aminotransferase (TAT; EC 2.6.1.5) was found in extracts of rosmarinic acid-producing cell cultures of *Anchusa officinalis* and *Coleus blumei*, while low activity of tyrosine oxidase was observed. During the culture cycle, TAT activity, phenylalanine ammonia-lyase (PAL) activity and the rate of rosmarinic acid synthesis changed in a coordinated manner. TAT from *A. officinalis* cultures displayed high substrate specificity for tyrosine but relatively broad specificity toward potential amino acceptors, with equal effectiveness for α-ketoglutarate and oxaloacetate. The enzyme activity was competitively inhibited by L-α-aminooxy-β-phenylpropionic acid (AOPP) with a K_i of 0.8×10^{-6} M. The block of the entry of tyrosine into the tyrosine-derived pathway by AOPP inhibition *in vivo* confirmed the role of TAT as the entrypoint enzyme of the pathway.

INTRODUCTION

Rosmarinic acid (α-*O*-caffeoyl-3,4-dihydroxyphenyllactic acid; RA), a plant secondary metabolite belonging to the class of hydroxycinnamic acid esters, is a prominent constituent of members of the Lamiaceae and Boraginaceae. Callus and suspension cultures have been successfully established from several plant species reported to accumulate RA, and some of these cultures, namely *Coleus blumei* [1, 2], *Anchusa officinalis* [3], *Lithospermum erythrorhizon* [4], *Ocimum basilicum* [5], *Salvia officinalis*, *Salvia triloba* and *Rosmarinus officinalis* [6] have been shown to continue producing the ester *in vitro*. Unlike some secondary metabolite-producing cultures [7, 8], the production of RA appears to be constitutively expressed in these cell lines without any special medium manipulation or selection pressure being required. Furthermore, some of these cultures, e.g. *C. blumei* and *A. officinalis*, accumulate almost exclusively RA, with a content higher than that of the parent plants. To better understand the molecular basis of selective expression of a specific metabolic sequence in cultured cell systems, a detailed study of the biochemistry of RA production has been undertaken.

Earlier studies of the biosynthesis of RA in *Mentha* [9] and later in *C. blumei* suspension cultures [1] showed that phenylalanine and tyrosine are the respective precursors of the caffeoyl and 3,4-dihydroxyphenyllactic acid units of RA. Since, in these studies, DOPA had also appeared to be able to serve as a precursor of the 3,4-dihydroxyphenyllactic acid moiety, hydroxylation of tyrosine at the 3-position had been proposed as the first step of the tyrosine-derived pathway. No tyrosine 3-hydroxylase activity could be detected in *Coleus* culture extracts, however [10]. Furthermore, blocking the flow through the phenylalanine-derived pathway by use of the phenylalanine ammonia-lyase (PAL) inhibitor α-aminooxy-β-phenylpropionate (AOPP) led to an accumulation of 4-hydroxyphenyllactic acid rather than DOPA [10]. These observations indicate that 4-hydroxyphenyllactate, not DOPA, is the more likely intermediate in the tyrosine-derived pathway, as shown in Fig. 1.

In the present study, this proposed pathway was evaluated by searching for the first enzyme of the pathway, which could be envisioned as either tyrosine aminotransferase or tyrosine oxidase. We have found that the enzyme catalysing the first step of the tyrosine-derived pathway of RA biosynthesis is probably tyrosine aminotransferase (TAT; EC 2.6.1.5).

RESULTS

Detection of tyrosine aminotransferase

Crude extracts prepared from linear growth phase *A. officinalis* cultured cells contained much higher activity of TAT than of tyrosine oxidase (Fig. 2). The transamination activity was linear with time for at least 30 min and was proportional to protein concentration between 10 and 125 μg protein per assay. Heat denatured enzyme was inactive. A similar pattern was also obtained with crude extracts from another high RA-producing cell culture, *Coleus blumei* (data not shown). The enzymic reaction product from both *Anchusa* and *Coleus* TAT preparations was identified as 4-hydroxyphenylpyruvate by co-chromatography (cellulose TLC; 10% HoAc; *n*-BuOH–HOAc–H_2O, 4:1:1) with the authentic compound.

Relationship of TAT and PAL activities to rate of RA synthesis

Figure 3 shows the changes with time in the specific activities of TAT and PAL and the rate of RA synthesis during a 16-day growth cycle of *Anchusa officinalis* cultures. Both TAT and PAL had a sharp maximum of activity at the same point in the late linear growth phase,

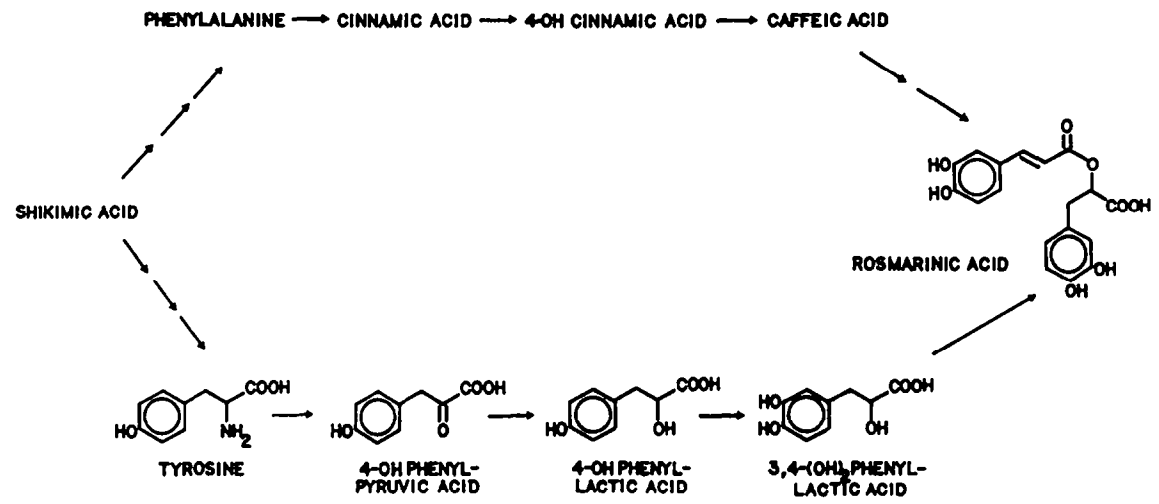

Fig. 1. Proposed pathway of rosmarinic acid biosynthesis.

although the increase of TAT activity occurred a few days before the rise in PAL activity. The rises in both enzyme activities also preceded the increase in the rate of RA synthesis which took place about one day after the increase in PAL activity. There thus appears to be a specific sequence followed in the expression of the two entrypoint enzymes at day 4–6 in the *Anchusa* culture cycle, which leads to a burst of RA synthesis. The subsequent rapid decline in both synthetic rate and enzyme activities, on the other hand, took place simultaneously and in parallel as the cultured cells entered stationary growth phase. A similar coordinated rise and fall in TAT and PAL activities was also observed in *C. blumei* cultures (Fig. 4).

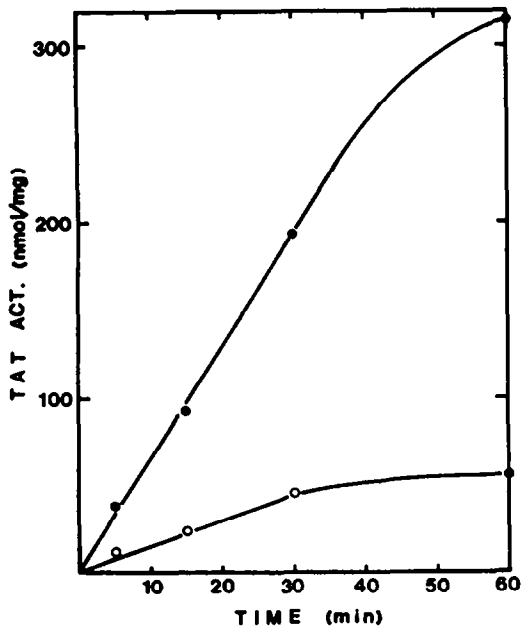

Fig. 2. Kinetics of the deamination of L-tyrosine by 7-day-old *Anchusa* crude extract under the standard assay conditions for tyrosine aminotransferase (●) and tyrosine oxidase (○).

Properties of tyrosine aminotransferase from A. officinalis *cell cultures*

Substrate specificity. Substrate specificity was examined using the partially purified *Anchusa* enzyme preparation. When activity was measured with various aromatic amino acids, using α-ketoglutarate as the amino acceptor (Table 1), it appeared that only L-tyrosine, and to a small extent L-phenylalanine, could be used as substrates. To determine the optimal amino acceptor, the rate of reaction of L-tyrosine in the presence of various α-keto acids was examined (Table 2). Both α-ketoglutarate and oxaloacetate were equally effective amino-acceptor substrates.

Effect of pH. The response of TAT activity to different buffers and pH values was studied by assaying in appropriate mixtures at 30°. As shown in Fig. 5, the enzyme was most active at around pH 9.0, and glycylglycine buffer appeared to support maximum TAT activity. The effect of pH on TAT stability was examined by preincubating the enzyme preparation in 50 mM Tris–HCl at different pH

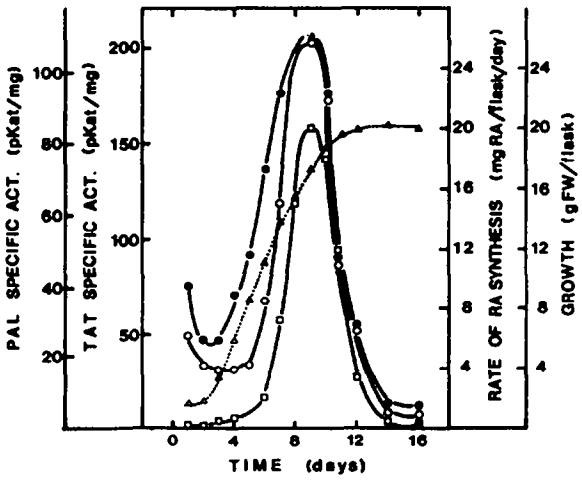

Fig. 3. Changes in TAT (●—●) and PAL (○—○) activities and rate of RA synthesis (□—□) during the growth cycle (△...△) of *A. officinalis*.

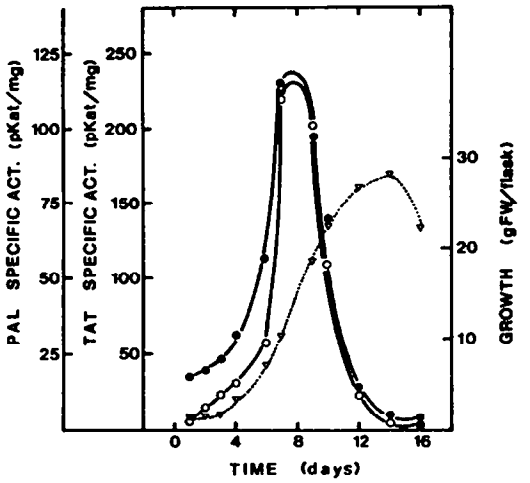

Fig. 4. Changes in TAT (●—●) and PAL (○—○) activities during the growth cycle (▽...▽) of *Coleus blumei* cell cultures.

Table 1. Activity of *Anchusa* TAT* in the presence of various aromatic amino acids and α-ketoglutarate

Aromatic amino acid	Relative activity (%)
L-Tyrosine	100
L-Phenylalanine	25
L-Tryptophan	0
L-DOPA	0

*The transamination of each substrate was measured spectrophotometrically as described in Experimental, except that the following wavelengths were used for quantitating the corresponding product: λ_{331} for L-tyr, λ_{320} for L-phe, λ_{328} for L-trp and λ_{335} for L-DOPA.

Table 2. Activity of *Anchusa* TAT in the presence of various α-keto acids and L-tyrosine

α-Keto acid (10 mM)	Relative activity %
α-Ketoglutarate	100
Oxaloacetate	100
Pyruvate	46
Glyoxylate	0
None	0

values, using two different pre-incubation treatments: 4° for 6 hr or 37° for 30 min. Enzyme activity was subsequently assayed at pH 9 and 30°. The enzyme was the most stable at pH 8.2 (Fig. 6).

Effect of polyhydric alcohols. Crude extracts prepared by using the usual ratio of 1 g fresh cells per 3 ml extraction medium contained relatively low protein concentrations (0.5–0.6 mg/ml). An examination of the effect

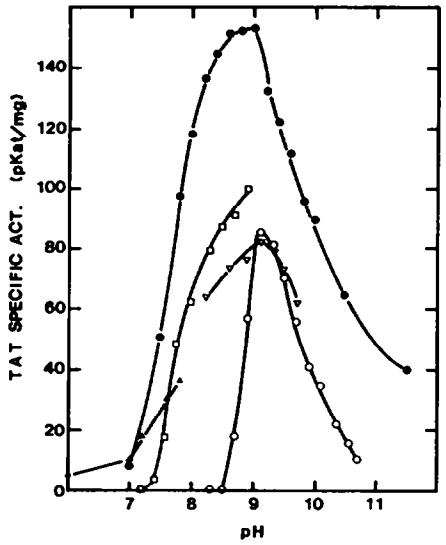

Fig. 5. Effect of pH on *Anchusa* TAT activity. The enzyme activity was assayed in the following buffers: 50 mM glycylglycine–KOH (●), 100 mM Tris–HCl (□), 100 mM Na^+ phosphate (△), 50 mM borate–KOH (○) and 100 mM Tricine–KOH (▽).

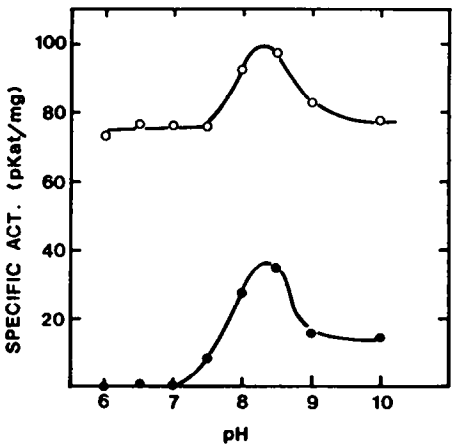

Fig. 6. Effect of pH on *Anchusa* TAT stability. The partially purified enzyme was stored at 4° for 6 hr (○) or 37° for 30 min (●) in 50 mM Tris–HCl adjusted to the pH values shown. TAT activity was then assayed under standard conditions.

of protein concentration on stability of TAT at 30° (Fig. 7) showed that the enzyme lost significant activity when the protein concentration was < 1.5 mg/ml. Inclusion of sucrose in the extraction buffer, however, resulted in both a stabilization of the activity at low protein concentration and an overall enhancement of TAT activity (Fig. 7). Glycerol was less effective than sucrose. The optimal sucrose concentration was found to be 150 mM (data not shown).

Effect of protease inhibitors. Inclusion of various protease inhibitors in the extraction medium did not significantly affect the extractable TAT activity when assayed immediately (Table 3). Upon prolonged incubation at 30°, however, considerable change in TAT activity was observed. As shown in Table 3, TAT activity in the crude

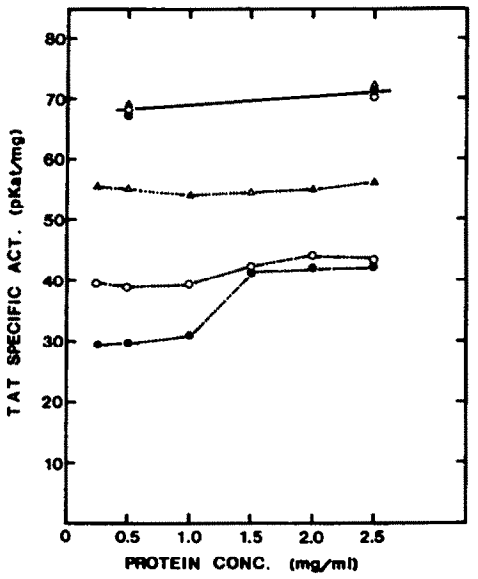

Fig. 7. Effect of protein concentration on *Anchusa* TAT stability. The partially purified enzyme at various concentrations was stored at −70° for 24 hr (△), 4° for 24 hr (○) or 30° for 30 min (●), in the presence (———) and absence (- - -) of 150 mM sucrose. TAT activity was then assayed under standard conditions.

Table 3. Effect of various protease inhibitors on TAT activity*

Compound (1 mM)	Relative activity† (%) Incubation time (hr) at 30° 0	2	4
None	100	178	140
EDTA	94	213	240
Leupeptin	89	149	139
NBS	94	169	199
*p*CMB	100	187	81
PMSF	87	166	188
TLCK	91	146	27

Abbreviations: NBS = *N*-bromosuccinimide, *p*CMB = *p*-chloromercuribenzoate, PMSF = phenylmethylsulphonyl fluoride, TLCK = α-*N*-tosyl-L-lysine chloromethyl ketone.

*Each protease inhibitor (1 mM) was included in the extraction buffer containing 50 mM Tris–HCl, pH 7.5, 150 mM sucrose and 5 mM β-mercaptoethanol. After enzyme extractions as described in Experimental, each crude extract was incubated at 30° for the period of time indicated, and then assayed for TAT activity.

†TAT activity is expressed as a percentage of the activity measured at 0 hr in the crude extract containing no inhibitors.

extract held at 30° for 4 hr was activated by EDTA, NBS or PMSF. The presence of PCMB or TLCK, on the other hand, apparently inhibited TAT activity. It should be noted that prolonged incubation of *Anchusa* crude extract in the absence of protease inhibitors also activated TAT activity to some extent. Measured TAT activities therefore reflect a combination of effects from the protease inhibitors, the temperature and possibly other, unknown, factors. For stabilization of TAT activity during enzyme purification, however, the inclusion of EDTA in the extraction and storage buffers appeared to be advantageous.

Effect of aminooxy substrate analogues. Pyridoxal phosphate-utilizing enzymes are often very sensitive to inhibition by compounds in which the usual amine function has been replaced by an aminooxy moiety [11]. Partially-purified TAT was found to be 74% inhibited by 1.0 mM aminooxacetate but the activity was far more sensitive to L-α-aminooxy-*p*-phenylpropionate (Fig. 8A). A double reciprocal plot of the kinetic data for the AOPP incubations was consistent with a competitive mode of inhibition and an apparent K_i of 0.8×10^{-6} M AOPP (Fig. 8B).

In vivo incubation of *A. officinalis* cells for 90 min with 0.5 mM AOPP effectively blocked incorporation of labelled L-tyrosine into RA. Examination of the distribution of the label among the ethanol-soluble metabolites showed that only labelled tyrosine could be detected in the inhibited cells, whereas the bulk of the label appeared in RA in the uninhibited cells (Fig. 9).

DISCUSSION

The results of the present study reveal that the first enzyme of the rosmarinic acid (RA) biosynthetic pathway is probably tyrosine aminotransferase rather than tyrosine oxidase. The presence of TAT activity in extracts of both *A. officinalis* and *C. blumei* cell cultures also indicates that the same route for conversion of tyrosine into RA is operating in both cultures. This conclusion is supported by time-course studies in both cultures where the TAT activity appeared and disappeared in a manner which suggests a coordination with the expression of PAL activity and consequently with the onset of RA synthesis (Figs 3 and 4). At present, the exact factors controlling the timing of expression of TAT and PAL are unknown, although it seems likely that some common, or closely linked, mechanisms must exist to regulate the activities of both enzymes. It is known from earlier work [3] that RA synthesis is initiated at a point in the culture cycle when some key macronutrients (phosphate, ammonium-N) have been depleted from the medium which may indicated that nutrient stress is triggering secondary metabolism.

The discovery of TAT activity in the RA-producing cell cultures allowed the properties of this enzyme to be examined *in vitro*. *Anchusa* TAT proved to be specific for L-tyrosine but displayed relatively broad specificity toward potential amino acceptors (Tables 1 and 2). The reaction pH optimum of 9.0 (Fig. 5) is higher than that reported for rat TAT (pH 7.5) [12] and for most plant aminotransferases (~ pH 8.0) [13–15], but is very similar to that of PAL (pH 8.8–9.0). This may reflect a degree of organization of the RA biosynthetic machinery which places TAT and PAL in the same microenvironment. For enzyme stabilization, pH 8.2 and the inclusion of sucrose and EDTA in both the extraction and storage buffers appeared to be optimal (Figs 6 and 7).

The potent inhibition of *Anchusa* TAT by AOPP is in contrast to the relatively weak effect reported for AOPP on mung bean aromatic aminotransferase [16]. This difference may be related to the high specificity of the *Anchusa* enzyme for phenylpropanoid substrates, or could be another example of interspecific variation. The latter phenomenon has recently been observed for

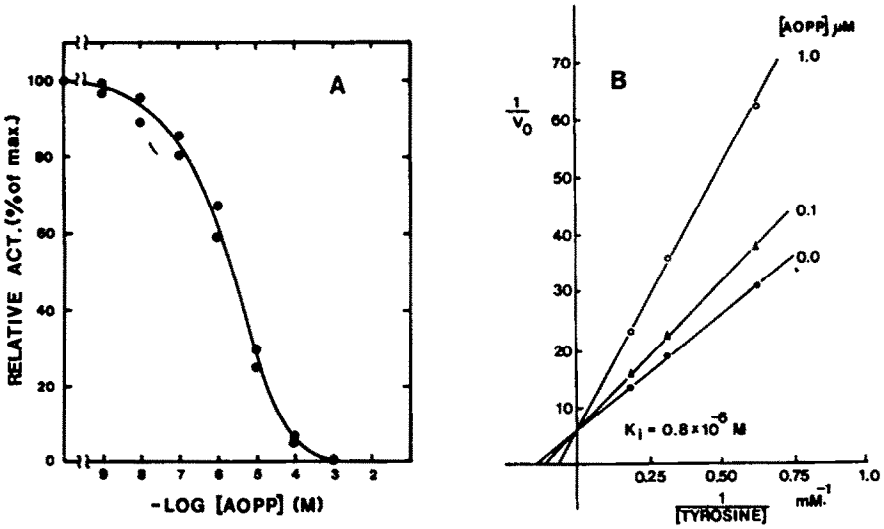

Fig. 8. (A) Inhibition of *Anchusa* TAT activity by AOPP. The enzyme activity was determined in the standard assay in the presence of different concentrations of AOPP. (B) Double-reciprocal plots of *Anchusa* TAT activity assayed in the presence of different substrate concentrations and 0 μM (●), 0.1 μM (▲) or 1.0 μM (○) AOPP.

another pyridoxal phosphate-utilizing enzyme (tyrosine decarboxylase) in a series of plant species, where sensitivity of the decarboxylase to AOPP ranged from strong inhibition to no effect [17].

The accumulation of labelled tyrosine in AOPP-inhibited *Anchusa* cultures differs from the earlier reported accumulation of label in 4-hydroxyphenyllactate in AOPP-inhibited *Coleus blumei* cultures [10]. This may be due to a lower sensitivity of the *Coleus* TAT toward AOPP, or to the different incubation conditions (30 min and 4 hr incubation for *Anchusa* and *Coleus* cultures, respectively) used in the two experiments. The results are nevertheless consistent with TAT serving as the entrypoint enzyme which channels tyrosine into RA synthesis. Whether the TAT activity described here is also responsible for conversion of 4-hydroxyphenylpyruvate to L-tyrosine during *de novo* biosynthesis of the latter within the shikimate pathway depends on the mode of tyrosine synthesis in this species. In the event that tyrosine in *Anchusa* is formed predominantly by way of arogenate as proposed for other plant species [21] the *Anchusa* TAT must be considered a specialized enzyme with a specific role in secondary metabolism. It is hoped that full purification and characterization of the enzyme will reveal whether it possesses regulatory properties appropriate for its metabolic situation.

EXPERIMENTAL

Cultures. Suspension cultures of *Anchusa officinalis* L. and *Coleus blumei* Benth. were established and characterized as described elsewhere [1, 3]. The cultures were maintained in B5 medium [18] containing 1.0 mg/l 2,4-D, 0.1 mg/l kinetin and 3% sucrose. Subculturing was carried out by a 1:8 dilution into 50 ml fresh medium (250 ml Erlenmeyer flask) at 7-day intervals. Flasks were aerated by 130 rpm gyratory shaking at 25° in continuous light. For time-course studies, cell growth and RA content were measured as previously described [3].

Enzyme preparation. Cultures were harvested by vacuum filtration on Miracloth discs, rinsed with distilled water, frozen in liquid N_2, and ground to a powder in a cold mortar. The powder was extracted by stirring for 20 min in three volumes of extraction buffer (50 mM Tris–HCl pH 7.5, 5 mM β-mercaptoethanol) containing buffer-soaked Polyclar AT powder 1.0 g/g tissue). After filtration through Miracloth and centrifugation (20 000 *g*; 10 min), the supernatant was desalted (Biogel P6-DG) and used as crude extract. For a partially purified TAT preparation, undesalted crude extract from seven-day-old *Anchusa* suspension cultures was fractionated by $(NH_4)_2SO_4$ precipitation (40–80% saturation). The protein was redissolved in a minimum vol. of extraction buffer and desalted as above.

Tyrosine aminotransferase. This was assayed by a modification of the method of Diamondstone [19]. This fixed-time assay depends upon the alkali-catalysed oxidation of 4-hydroxyphenylpyruvate to 4-hydroxybenzaldehyde and oxalate. The standard assay mixture contained 6.0 μmol L-tyrosine, 10 μmol α-ketoglutarate, 0.05 μmol pyridoxal phosphate and 42.5 μmol glycylglycine (pH 9.0), in a total vol. of 1.0 ml. The reaction was started by the addition of 50 μl enzyme and allowed to run at 30° for 30 min. It was stopped by the addition of 20 μl 10 M KOH, with rapid mixing. The absorbance of the soln was read against a reagent blank at 331 nm. Under these conditions, the extinction coefficient of the product (4-hydroxybenzaldehyde) is 24 900 M^{-1}, but since only 80% of the 4-hydroxyphenylpyruvate is converted to 4-hydroxybenzaldehyde, an effective extinction coefficient of 19 900 M^{-1} was used [19]. Enzyme specific activity is expressed in pkat/mg of protein.

Tyrosine oxidase. Activity was determined by radioassay. The reaction mixture contained 3.25 μmol L-tyrosine (0.1 μCi L-[U-^{14}C]tyrosine), 70 μmol KCl and 88.5 μmol potassium phosphate, pH 7.6, in a total volume of 0.8 ml. The reaction was started by the addition of 100 μl enzyme. After 0, 5, 15, 30 and 60 min incubation at 30°, it was stopped by addition of 70 μl conc. HCl. Following addition of 20 μl 0.5 M unlabelled 4-hydroxyphenylpyruvate as carrier, radioactive product was extracted into 2.5 ml toluene–EtOAc (1:1). Tubes were vortexed for 20 sec and the two phases separated by centrifugation (1 min; 1250 *g*). Production of radioactive 4-hydroxyphenylpyruvate was quantitated by scintillation counting of 1.0 ml of the organic phase in 5.0 ml scintillation cocktail.

Phenylalanine ammonia-lyase. Activity was determined by radioassay. The standard assay mixture contained 1.5 μmol L-

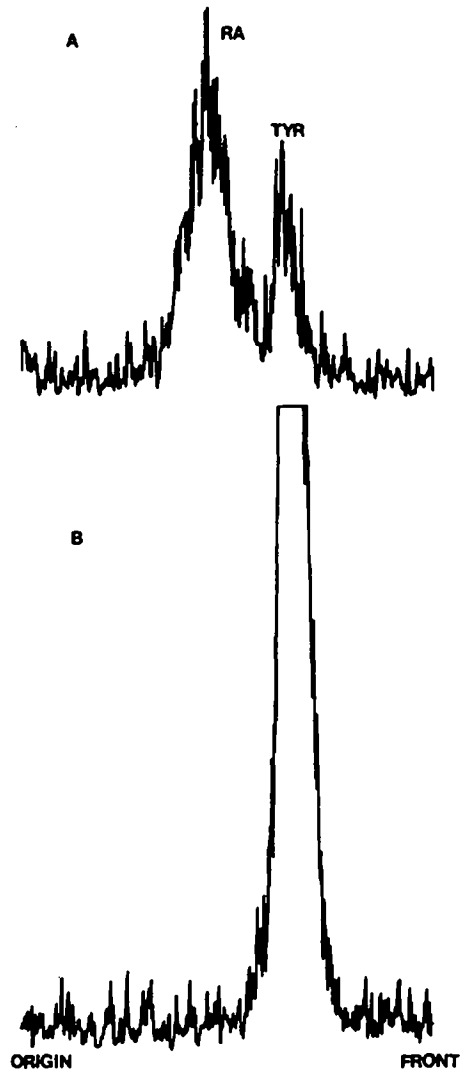

Fig. 9. Radioactivity profiles for TLC chromatogram of ethanol-soluble metabolites prepared from 7-day-old *Anchusa* cell cultures fed L-[U-^{14}C]tyrosine in the absence (A) and presence (B) of 0.5 mM AOPP.

phe (0.3 μCi L-[2,6-^{3}H]phenylalanine), 6 μmol β-mercaptoethanol and 60 μmol potassium borate, pH 8.7, in a total volume of 1.5 ml. The reaction was started by the addition of 200 μl enzyme. After 30 min incubation at 30°, 100 μl 5.0 M HCl and 20 μl 0.5 M cinnamic acid (in EtOH) were added. The radioactive product was then extracted and quantitated as described in the tyrosine oxidase assay.

Protein assay. Protein concentration was determined by the Coomassie Brilliant Blue G-250 dye-binding assay [20] using BSA as a reference protein.

In vivo *inhibition of TAT activity.* Feeding experiments were conducted using 10 ml of a 7-day-old *A. officinalis* cell suspension (0.5 g tissue) in 50 ml screw-top jars shaken at 130 rpm. After 1 hr preincubation with 0.5 mM AOPP (control incubations contained no AOPP), the cells were supplied with 0.1 μmol L-[U-^{14}C]tyrosine (3.0×10^6 dpm) and incubated for a further 30 min. The harvested cells were rinsed, extracted in hot 70% EtOH (50 ml) and the extract concentrated to an aqueous residue (1 ml). Aliquots of the residue were then fractionated by TLC (cellulose; 10% HOAc). The distribution of radioactivity on the TLC plate was measured by scanning with a windowless gas-flow detector.

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