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The Aromatic Amino Acid Pathway Branches at L-Arogenate in *Euglena gracilis*

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The recently characterized amino acid L-arogenate (Zamir et al., J. Am. Chem. Soc. **102**:4499–4504, 1980) may be a precursor of either L-phenylalanine or L-tyrosine in nature. *Euglena gracilis* is the first example of an organism that uses L-arogenate as the sole precursor of both L-tyrosine and L-phenylalanine, thereby creating a pathway in which L-arogenate rather than prephenate becomes the metabolic branch point. *E. gracilis* ATCC 12796 was cultured in the light under myxotrophic conditions and harvested in late exponential phase before extract preparation for enzymological assays. Arogenate dehydrogenase was dependent upon nicotinamide adenine dinucleotide phosphate for activity. L-Tyrosine inhibited activity effectively with kinetics that were competitive with respect to L-arogenate and noncompetitive with respect to nicotinamide adenine dinucleotide phosphate. The possible inhibition of arogenate dehydratase by L-phenylalanine has not yet been determined. Beyond the latter uncertainty, the overall regulation of aromatic biosynthesis was studied through the characterization of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase and chorismate mutase. 3-Deoxy-D-*arabino*-heptulosonate 7-phosphate synthase was subject to noncompetitive inhibition by L-tyrosine with respect to either of the two substrates. Chorismate mutase was feedback inhibited with equal effectiveness by either L-tyrosine or L-phenylalanine. L-Tryptophan activated activity of chorismate mutase, a pH-dependent effect in which increased activation was dramatic above pH 7.8. L-Arogenate did not affect activity of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase or of chorismate mutase. Four species of prephenate aminotransferase activity were separated after ion-exchange chromatography. One aminotransferase exhibited a narrow range of substrate specificity, recognizing only the combination of L-glutamate with prephenate, phenylpyruvate, or 4-hydroxyphenylpyruvate. Possible natural relationships between *Euglena* spp. and fungi previously considered in the literature are discussed in terms of data currently available to define enzymological variation in the shikimate pathway.

Euglenoid microorganisms are photosynthetic eucaryotes of exceedingly diverse morphology. They are reminiscent in morphology of protozoa and algal flagellates. Like green algae and plants, they contain chlorophyll species *a* and *b* (7). With respect to variant enzymological patterning of biochemical pathways, euglenoids have been grouped with the higher fungi, primarily because both groups use the L-α-aminoadipic acid pathway for lysine biosynthesis. In contrast, bacteria, algae, and plants all use the diaminopimelate pathway for lysine biosynthesis. Detailed biochemical studies with euglenoids have been carried out primarily with *Euglena gracilis*. A striking resemblance between *E. gracilis* and *Neurospora crassa* (a higher fungus) was found in their common formation of a single multifunctional protein catalyzing steps 2 to 6 in the shikimate pathway of aromatic biosynthesis (2). However, when enzymes of tryptophan biosynthesis in *E. gracilis* were compared with those of higher fungi, a unique arrangement of enzyme aggregation was found in *E. gracilis* (5). On the other hand, the general observation that tryptophan pathway enzymes are aggregated in some combination or other among euglenoids and higher fungi could be regarded as a general similarity.

As with lysine, two variant routes exist in nature for biosynthesis of L-phenylalanine and L-tyrosine (see Fig. 1). Thus far, most attention has focused on procaryotic organisms. The use of the 4-hydroxyphenylpyruvate route for L-tyrosine biosynthesis has been extensively documented in many microorganisms (18). Organisms that exclusively use the alternative route

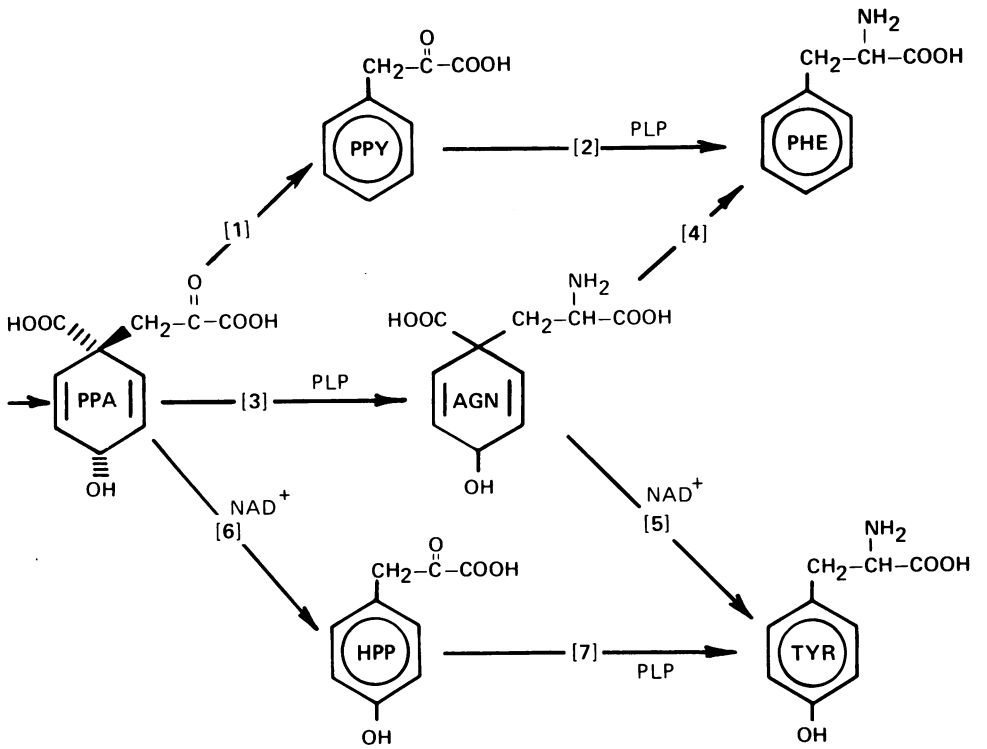

FIG. 1. *Variant biochemical pathways to* L-*phenylalanine and* L-*tyrosine in nature. The dual routings as shown are illustrative of P. aeruginosa (27). Microorganisms are known which express any combination of the one or two pathways to the end product amino acids. The dehydrogenase activities shown, although NAD linked in P. aeruginosa, may be NADP linked in other organisms. Abbreviations: PPA, prephenate; AGN,* L-*arogenate; PPY, phenylpyruvate; HPP, 4-hydroxyphenylpyruvate; PHE,* L-*phenylalanine; TYR,* L-*tyrosine; PLP, pyridoxal 5'-phosphate. Enzymes are numbered as follows: [1] prephenate dehydratase; [2] phenylpyruvate aminotransferase; [3] prephenate aminotransferase; [4] arogenate dehydratase; [5] arogenate dehydrogenase; [6] prephenate dehydrogenase; and [7] 4-hydroxyphenylpyruvate aminotransferase.*

(via the amino acid intermediate arogenate) include the cyanobacteria (33) and coryneform species of bacteria (12, 14). In addition, a large number of *Pseudomonas* and *Alcaligenes* species have been shown to possess dual pathways to L-tyrosine (8, 27). For L-phenylalanine biosynthesis the most common pathway appears to be the traditional phenylpyruvate route (13, 16, 20, 22). In *Pseudomonas aeruginosa* enzymes of both the phenylpyruvate route and the alternative arogenate route have been characterized (27, 28). Prior to the current results obtained with *E. gracilis*, no organism has been shown to rely exclusively upon arogenate as an obligatory precursor of both L-phenylalanine and L-tyrosine.

Previous studies on the shikimate pathway in *E. gracilis* have demonstrated 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthase activity (36), enzymes of the tryptophan branch (24), activity of the aromatic multienzyme aggregate catalyzing six enzymes in the prechorismate section of the pathway (26), and chorismate mutase (37). Prephenate dehydratase and prephenate dehydrogenase were reported to be absent in a 3,000-fold-purified preparation of chorismate mutase, and it appears that these activities were not found in crude extracts. Indeed, preliminary studies of our own did not reveal these activities in crude extracts of *E. gracilis*. These observations can now be accounted for by the obligate use of the intermediate arogenate as the sole precursor of L-phenylalanine and L-tyrosine biosynthesis via the enzymes arogenate dehydratase and arogenate dehydrogenase. These enzymological results are qualitatively different from those obtained in *N. crassa*.

MATERIALS AND METHODS

Growth of organisms and preparation of extracts. *E. gracilis* ATCC 12796 was purchased from

the American Type Culture Collection, Rockville, Md. Cultures were grown in the light on euglena broth (Difco Laboratories, Detroit, Mich.) and harvested by centrifugation. The cell pellet obtained was resuspended in 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM dithiothreitol, and the cells were disrupted by sonic oscillation at 4°C (Lab-line Ultratip, Labsonic System, Melrose Park, Ill.). Cellular debris was removed by centrifugation at 150,000 × *g* for 1 h. Extracts were subsequently passed through a Sephadex G-25 column at 4°C to remove small molecules.

Analytical procedures. (i) Protein measurements. Concentration of protein in extracts was estimated by the method of Bradford (6) as described in Bio-Rad technical bulletin 1051 (Bio-Rad Laboratories, Richmond, Calif.).

(ii) Enzyme assays. Enzyme assays were carried out with saturating substrate and under conditions of proportionality with respect to time and protein concentration. With arogenate dehydratase, reaction velocities were proportional functions of time for 15 min, using 0.4 mg of protein per reaction mixture. Higher protein concentrations or longer incubation times led to sharply decreased velocities. Prephenate and arogenate dehydrogenase were assayed by following reduced nicotinamide adenine dinucleotide phosphate (NADPH) formation as previously described (8). With arogenate dehydrogenase assays, the formation of L-tyrosine from L-arogenate was confirmed and quantitated by a technique involving dansylation and thin-layer chromatography (14). Chorismate mutase and prephenate dehydratase were assayed by the method of Patel et al. (27). Arogenate dehydratase was assayed by modification (32a) of the fluorescent phenylalanine assay (Sigma technical bulletin 60F, Sigma Chemical Co., St. Louis, Mo.). The method of Calhoun et al. (9) was used to assay DAHP synthase. Aminotransferase enzymes were assayed by an adaptation (15) of the Miller and Thompson method (25). A mixed-substrate assay (31) was used to maximize the detection of multiple species of aminotransferase enzymes eluted from diethylaminoethyl (DEAE) cellulose columns. The reaction mixture contained: 0.125 mM bovine serum albumin; 25 μM pyridoxal 5'-phosphate; 1.0 mM each potassium prephenate, 4-hydroxyphenylpyruvate, phenylpyruvate, and 2-ketoglutarate; and 20.0 μl of a 1 mM ^{14}C-labeled L-amino acid mixture (phenylalanine, tyrosine, leucine, glutamate, and aspartate).

Column chromatography. Desalted crude extract preparations (100 mg of protein) were applied to a DEAE-cellulose (Whatman DE52) column (1.5 by 20 cm) equilibrated with 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM dithiothreitol. The column was washed with 100 ml of equilibration buffer, and a 300-ml linear salt gradient (0 to 0.7 M KCl or 0 to 0.3 M KCl) in the same buffer was applied. Fractions of 2.2 ml were collected.

Biochemicals and chemicals. Amino acids, keto acids, NAD, NADP, and Sephadex G-25 were obtained from Sigma Chemical Co. DEAE-cellulose (DE-52) was obtained from Whatman (Chilton, N.J.). Liquifluor and radioactive amino acids were from New England Nuclear Corp., Boston, Mass. and Amersham Corp., Arlington Heights, Ill., respectively. We used the sulfate salts of all divalent cations tested with DAHP synthase. Barium prephenate was prepared from culture supernatants of a tyrosine auxotroph of *Salmonella typhimurium* (10) and was converted to the potassium salt with excess K_2SO_4 before use. Chorismate was prepared from culture supernatants of *Klebsiella pneumoniae* according to the method of Gibson (17). L-Arogenate was prepared from culture supernatants of a triple auxotroph of *N. crassa* (23). The purification and isolation were modified according to the method of Zamir et al. (39). Prephenate preparations, free of detectable arogenate or phenylpyruvate, were about 90% pure. L-Arogenate preparations, free of detectable prephenate, phenylpyruvate, or phenylalanine, were 90 to 95% pure.

RESULTS

Absence of prephenate dehydrogenase and prephenate dehydratase. When crude extracts of *E. gracilis* were assayed for key enzymes of aromatic amino acid biosynthesis, activities were readily found for DAHP synthase, shikimate dehydrogenase, and chorismate mutase (Table 1). No activity was found for prephenate dehydrogenase (in combination with

TABLE 1. *Enzyme activities of aromatic amino acid biosynthesis detected in crude extracts*[a]

Enzyme assayed	Pathway position[b]	Presence[c]	Sp act (nmol/min per mg of protein)
DAHP synthase	[1]	+	22.4
Shikimate dehydrogenase	[4]	+	30.7
Chorismate mutase	[8]	+	32.9
Prephenate aminotransferase	[9]	+	25.7
Prephenate dehydrogenase[d]		−	
Prephenate dehydratase		−	
Arogenate dehydratase	[10]	+	0.6
Arogenate dehydrogenase[e]	[11]	+	350

[a] Extracts were prepared from myxotrophically grown cells harvested in the late exponential phase of growth as described in the text.

[b] Position of enzyme in the pathway corresponds to the numbering shown in Fig. 10.

[c] +, Presence of activity; −, absence.

[d] Assayed with NAD and NADP.

[e] Assayed with NADP; NAD did not substitute for NADP.

either NAD or NADP) or for prephenate dehydratase, however. No data have been reported for these enzymes in the literature. The presence of excellent prephenate aminotransferase activity (Table 1) posed the possibility that arogenate dehydrogenase and arogenate dehydratase might complete the enzymatic sequence to L-tyrosine and L-phenylalanine, respectively. These activities were indeed found in the crude extract preparations.

Characterization of partially purified enzymes. DAHP synthase, shikimate dehydrogenase, chorismate mutase, and arogenate dehydrogenase all separated from one another after elution from DEAE-cellulose (Fig. 2). Since shikimate dehydrogenase is an activity of a multifunctional protein (26), its position represents the position of enzymes 2 to 6 of the shikimate trunk. The elution profile of arogenate dehydratase was coincident with that of arogenate dehydrogenase (data not shown to simplify the graph).

(i) Arogenate/NADP dehydrogenase. Arogenate/NADP dehydrogenase was found to be

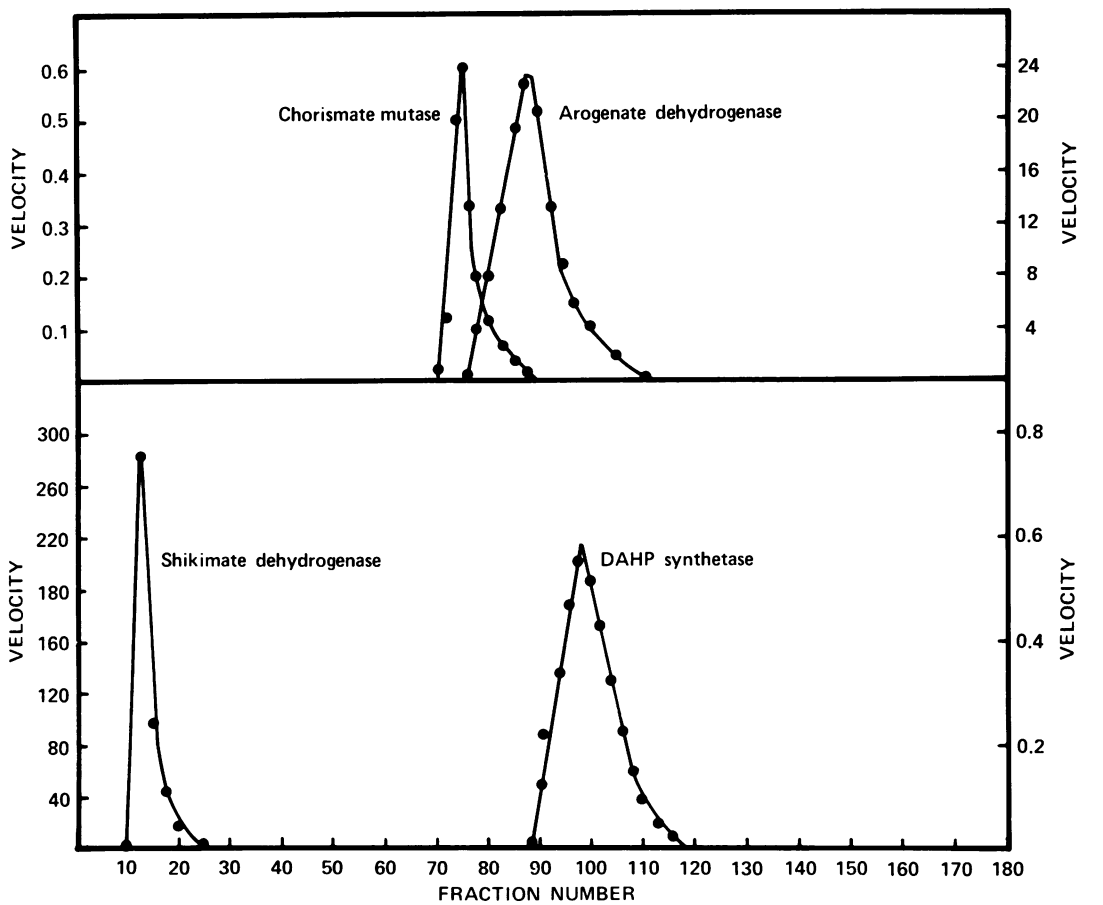

FIG. 2. *Fractionation of aromatic pathway enzymes after DEAE-cellulose chromatography. Velocities on the right and left ordinate scales are expressed as change in fluorescence per minute of NADPH (excitation at 340 nm, emission at 460 nm) in the case of the two dehydrogenase enzymes, change in absorbance at 549 nm in the colorimetric assay for DAHP synthase, and change in absorbance at 320 nm for chorismate mutase. The peak profile of arogenate dehydratase (data points not shown) was coincident with that of arogenate dehydrogenase. The column was loaded with a crude extract having the specific activities shown in Table 1. The first 45 fractions represent the 100-ml wash eluate before salt gradient elution. The following specific activities of peak eluate fractions were obtained (nanomoles per minute per milligram of protein): DAHP synthase (562), shikimate dehydrogenase (99.1), chorismate mutase (184), arogenate dehydrogenase (985), and arogenate dehydratase (1.7). Chorismate mutase eluted at 0.19 M KCl, arogenate dehydrogenase (and arogenate dehydratase) eluted at 0.25 M KCl, and DAHP synthase eluted at 0.32 M KCl.*

exceedingly sensitive to feedback inhibition by L-tyrosine. Figure 3 illustrates the first-order kinetics of inhibition observed. An L-tyrosine concentration of <15 μM inhibited activity by 50% at saturating substrate concentrations. Since the kinetics of L-tyrosine inhibition are competitive with respect to L-arogenate (Fig. 4), the sensitivity to feedback inhibition at a limiting substrate concentration is considerable. The apparent K_m of arogenate dehydrogenase for arogenate was 0.2 mM, and that for NADP was 3.8 μM. Feedback inhibition by L-tyrosine was noncompetitive with respect to NADP. A K_i value of 4.5 μM L-tyrosine was calculated.

(ii) Arogenate dehydratase. Arogenate dehydratase coeluted with arogenate dehydrogenase, but was assayed without interference in the absence of NADP, essential for the dehydrogenase. Arogenate dehydratase activity was unaffected by L-tyrosine. Determination of the possible sensitivity of arogenate dehydratase to feedback inhibition by L-phenylalanine is technically difficult since the assay involves measurement of phenylalanine product formed. In several systems prephenate dehydratase activity is stabilized or activated, or both, by L-leucine, L-methionine, or L-tyrosine (13, 29, 30). The assay for prephenate dehydratase in the presence of these potential activators (1 mM) did not reveal activity. The presence of 10% (vol/vol) glycerol in the buffer before sonication, in the presence or absence of potential activators, did not result in the detection of prephenate dehydratase (or prephenate dehydrogenase) activity.

(iii) DAHP synthase. In crude extracts the activity of DAHP synthase deviated from proportionality rather dramatically at higher protein concentrations (Fig. 5). At high protein concentrations total inhibition was found. All activity data shown in this paper were obtained under conditions of proportionality. In crude extracts tyrosine inhibited DAHP synthase, fitting a unimetabolic pattern of control that is commonly found in nature (21). More detailed studies were carried out with partially purified DAHP synthase (Fig. 2). The high sensitivity of arogenate dehydrogenase to feedback inhibition (thus favoring intracellular accumulation of arogenate) suggested the possibility that a pattern of se-

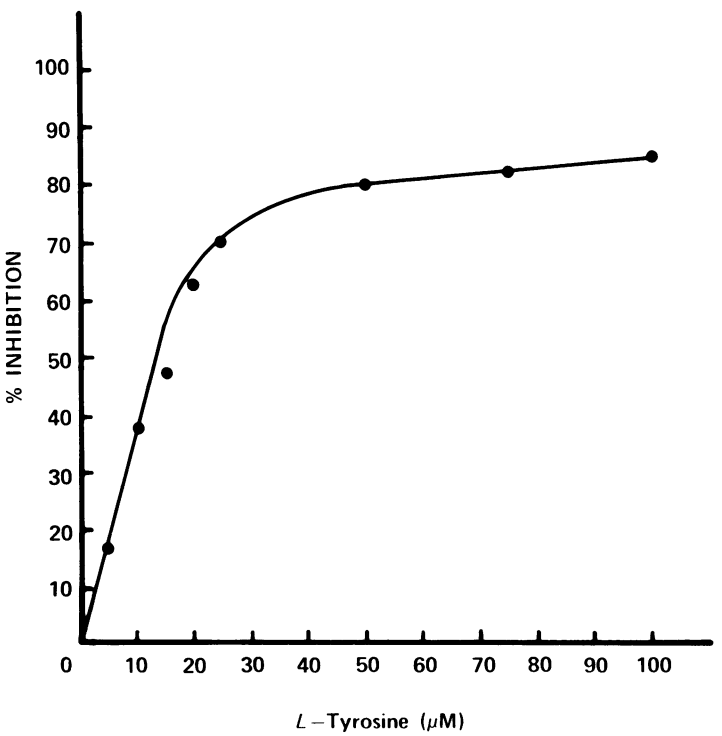

FIG. 3. *Inhibition curve showing effect of* L*-tyrosine upon activity of arogenate dehydrogenase. The enzyme assay was carried out as described in the text, using the partially purified preparation recovered in the experiment shown in Fig. 1.*

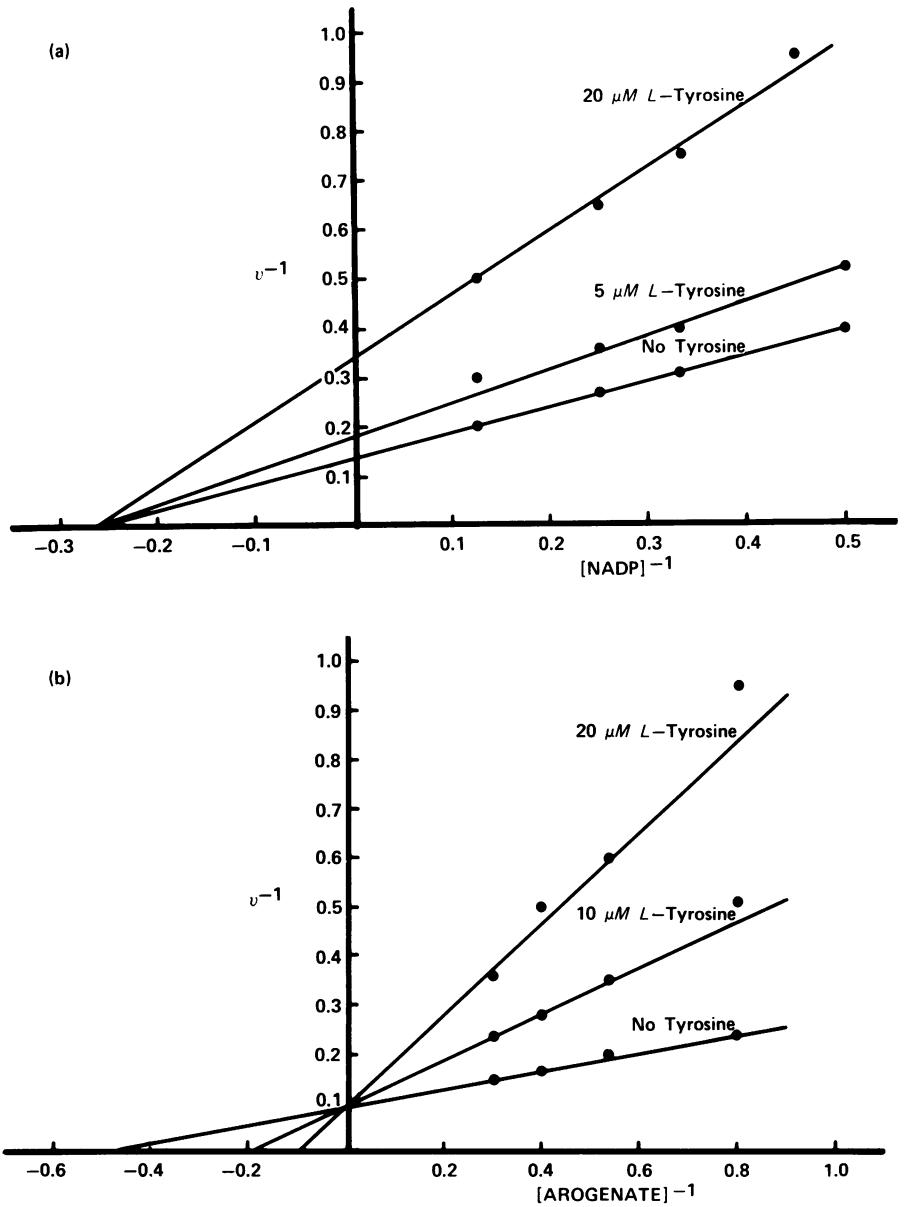

FIG. 4. *Double-reciprocal plots showing kinetic parameters of arogenate dehydrogenase. Partially purified enzyme recovered from the experiment shown in Fig. 1 was used. (a) Noncompetitive kinetics of tyrosine inhibition with respect to the variable substrate, NADP, when* L*-arogenate was fixed at a concentration of 375 μM. (b) Competitive kinetics of tyrosine inhibition with respect to* L*-arogenate as the variable substrate. The fixed substrate, NADP, was present at 0.5 mM.*

quential feedback inhibition, analogous to that exerted upon DAHP synthase by prephenate (19) in *Bacillus subtilis*, might exist in *Euglena* spp. Thus, L-arogenate might inhibit DAHP synthase or chorismate mutase or both. However, neither activity proved to be sensitive to inhibition by L-arogenate. The single species of DAHP synthase eluted (Fig. 2) was sensitive to feedback inhibition by L-tyrosine (Fig. 6), but not by L-phenylalanine or L-tryptophan. At saturating

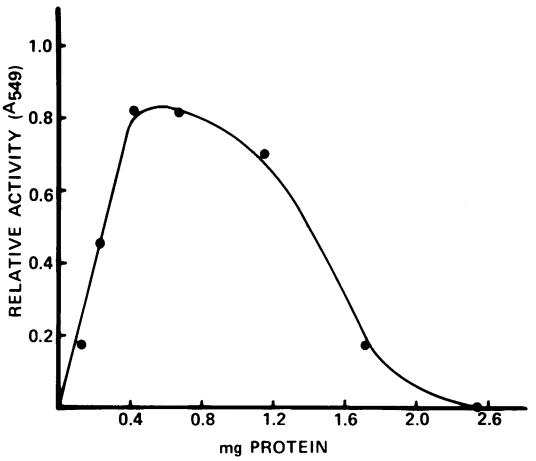

FIG. 5. *Relationship of DAHP synthase activity and protein concentration in crude extracts. A relative activity of 1.0 corresponds to a specific activity of 22.4 nmol of DAHP per min per mg of protein in crude extracts. Reactions were 20 min in elapsed time. A_{549}, Absorbance at 549 nm.*

substrate concentrations, 50% inhibition was achieved at about 16 μM L-tyrosine. The failure of the combination of aromatic amino acids (Fig. 6) to inhibit activity more than did L-tyrosine alone demonstrates the lack of a synergistic pattern of inhibition. The presence of certain divalent cations was stimulatory. Cobalt at a 1 mM concentration increased activity by 47%. Mg^{2+} or Mn^{2+} produced a lesser stimulation. The pH optimum for catalysis was 7.0 in potassium phosphate buffer. The apparent K_m for erythrose 4-phosphate was 0.50 mM; the apparent K_m for phosphoenolypyruvate was 0.67 mM. The inhibition of activity by L-tyrosine was noncompetitive with respect to each of the two substrates (Fig. 7).

(iv) Chorismate mutase. Chorismate mutase was also recovered from DEAE-cellulose as a single species. It was feedback inhibited by L-tyrosine and L-phenylalanine and was activated by L-tryptophan. At pH 7.5 and in the presence of chorismate at a saturating concentration, 0.5 mM L-phenylalanine inhibited activity by 75%, 0.5 mM L-tyrosine inhibited activity by 74%, and 0.5 mM L-tryptophan activated activity by 25%. The pH optimum for catalysis exhibits a broad range between 6.6 and 7.4, and a sharp decline occurs between pH 7.5 and 8.5 (Fig. 8). In the presence of L-tryptophan, however, the optimum was extended through a pH of at least 9.0. Thus, at pH 8.4 the addition of L-tryptophan to chorismate mutase stimulates activity 100-fold. In contrast, the action of the two inhibitor molecules is not pH responsive.

(v) Prephenate aminotransferase species. Aminotransferase enzymes capable of reaction with prephenate, phenylpyruvate, or 4-hydroxyphenylpyruvate were screened with a mixed-substrate assay (see Materials and Methods) to detect activities acting with different combinations of amino acid and keto acid substrates. Four species of aminotransferase enzymes were separated and designated as species AT-a, AT-b, AT-c, and AT-d (Fig. 9). All four enzymes were capable of transaminating prephenate, phenylpyruvate, or 4-hydroxyphenylpyruvate in the presence of an appropriate amino donor reactant (Table 2). AT-a exhibited the broadest range of activity with different substrate combinations. AT-a and AT-c were distinctly most active with the substrate combination of L-leucine and 2-ketoglutarate. AT-d was active only with L-glutamate in combination with prephenate, phenylpyruvate, or 4-hydroxyphenylpyruvate. AT-d is the most feasible aminotransferase candidate for functioning in vivo as prephenate aminotransferase. Although AT-d eluted very near chorismate mutase, the profiles (data not shown) were not coincident.

DISCUSSION

Obligatory role of L-arogenate as a precursor of L-tyrosine and L-phenylalanine. In nature, at least two alternative pathways

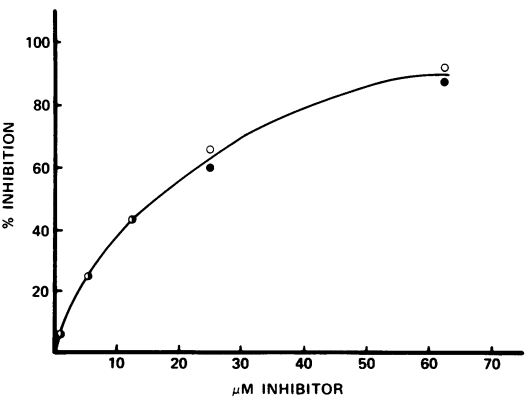

FIG. 6. *Inhibition of DAHP synthase activity by L-tyrosine (●). A mixture (○) of L-tyrosine, L-phenylalanine, and L-tryptophan (each at the concentrations specified on the abscissa scale) was identical to L-tyrosine alone in degree of inhibition produced. Data were obtained under assay conditions of proportionality, using partially purified enzyme recovered as shown in Fig. 2.*

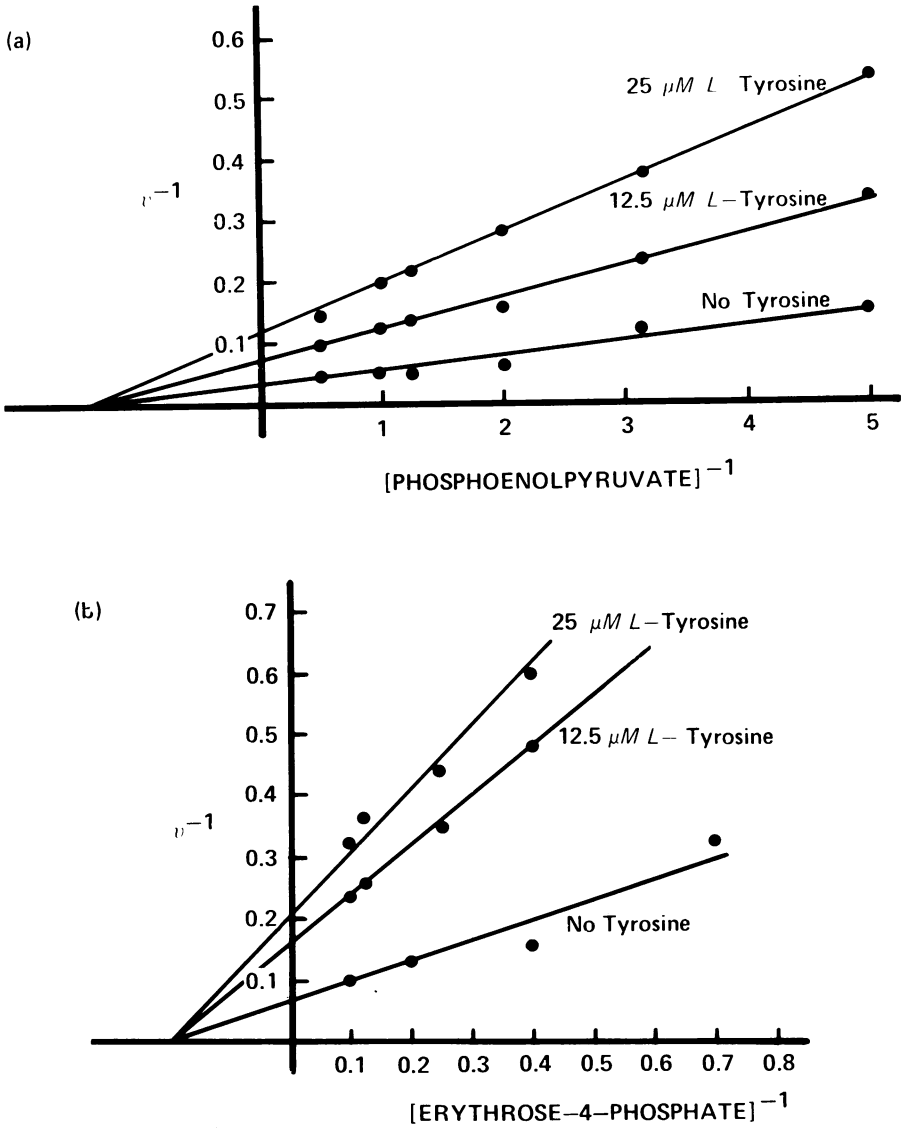

FIG. 7. *Double-reciprocal plots showing noncompetitive kinetics of inhibition of DAHP synthase by* L-*tyrosine with respect to: (a) phosphoenolpyruvate as the variable substrate, erythrose-4-phosphate fixed at 1.0 mM; and (b) erythrose-4-phosphate as the variable substrate, phosphoenolpyruvate fixed at 1.0 mM. Partially purified enzyme recovered as shown in Fig. 2 was used.*

intervene between prephenate and L-phenylalanine, either the phenylpyruvate route or the arogenate route. Likewise, either the 4-hydroxyphenylpyruvate route or the arogenate route may be used for L-tyrosine biosynthesis. In *P. aeruginosa* both sets of dual routings are present simultaneously (27). Virtually all possible combinations of pathways have been found. Thus far, *E. gracilis* is the only organism found to depend upon arogenate as the sole precursor of both phenylalanine and tyrosine. Accordingly, the metabolic branch point is located at arogenate in *Euglena* spp., instead of at prephenate as is the case in many organisms.

A highly variable feature of L-tyrosine biosynthesis in nature is the cofactor specificity of prephenate dehydrogenase and arogenate dehydrogenase. Examples of NAD-dependent and

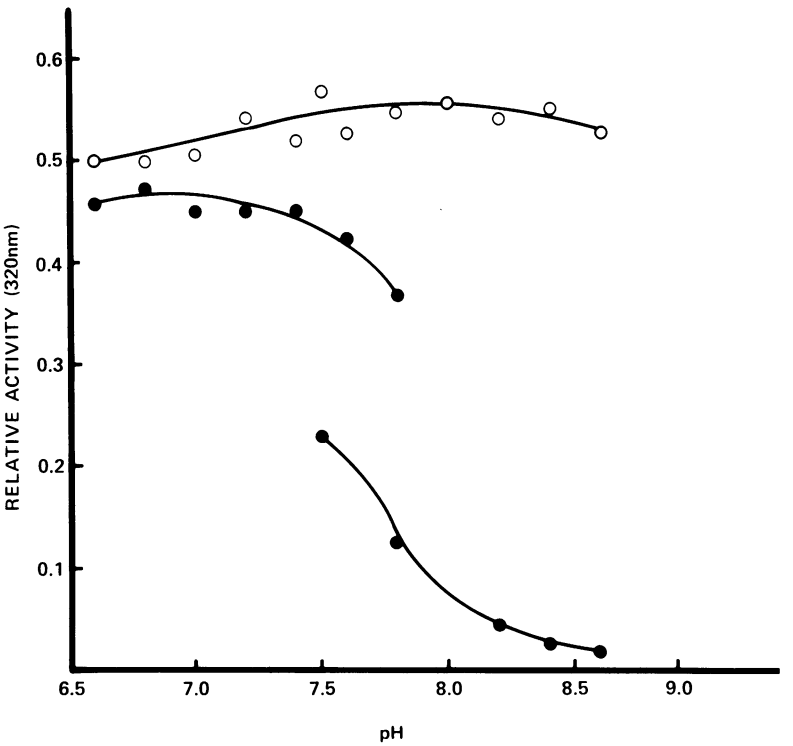

FIG. 8. *Effect of pH on partially purified chorismate mutase (recovered as shown in Fig. 2) in the presence* (○) *or absence* (●) *of 0.5 mM* L-*tryptophan. In the pH range of 6.6 to 7.7, potassium phosphate buffer was used; in the pH range of 7.5 to 8.6 Trizma-hydrochloride buffer was used. A relative activity of 0.1 absorbance unit at 320 nm corresponds to a specific activity of 4.7 nmol/min per mg of protein.*

NADP-dependent dehydrogenase have been described (8); it is also not uncommon to find examples of enzyme capable of functioning with either cofactor. In *E. gracilis* arogenate dehydrogenase is specific for NADP. Shoots of mung bean (*Vigna radiata*) also posess a NADP-specific arogenate dehydrogenase (31). This contrasts with arogenate dehydrogenase of corn, which is specific for NAD (G. S. Byng, R. J. Whitaker, C. E. Flick, and R. A. Jensen, Phytochemistry, in press)

L-Arogenate is formed via transamination of prephenate. As in *Brevibacterium flavum* (15) and *P. aeruginosa* (28), multiple species of aminotransferase active with prephenate as a keto substrate are found in *E. gracilis*. Although each of four aminotransferases is capable of functioning as prephenate aminotransferase in vitro, species AT-d probably plays at least a dominant role in vivo. This aminotransferase is the most specific, requiring L-glutamate as the amino donor reactant. Species AT-d is also capable of transaminating both phenylpyruvate and 4-hydroxyphenylpyruvate in vitro, although these keto acids are presumably not generated in vivo.

Overall regulation of the aromatic pathway in *Euglena* spp. The general picture of regulatory features which characterize aromatic amino acid biosynthesis in *Euglena* spp. are diagrammed in Fig. 10. The initial pathway catalyst, DAHP synthase, is feedback inhibited by L-tyrosine. This pattern of control has been denoted as the retro-tyr variation of unimetabolite control (21). L-Tryptophan is an excellent feedback inhibitor of anthranilate synthase, the first committed step of tryptophan biosynthesis. Chorismate mutase exhibits a balanced allosteric responsivity to L-phenylalanine and L-tyrosine as inhibitors; L-tryptophan is an allosteric activator. L-Arogenate showed no tendency to mimick L-tyrosine as an inhibitor of either DAHP synthase or chorismate mutase. L-Tyrosine is an effective inhibitor of arogenate dehydrogenase. It is uncertain thus far whether arogenate dehydratase is sensitive to inhibition by phenylalanine (owing to technical difficulties in

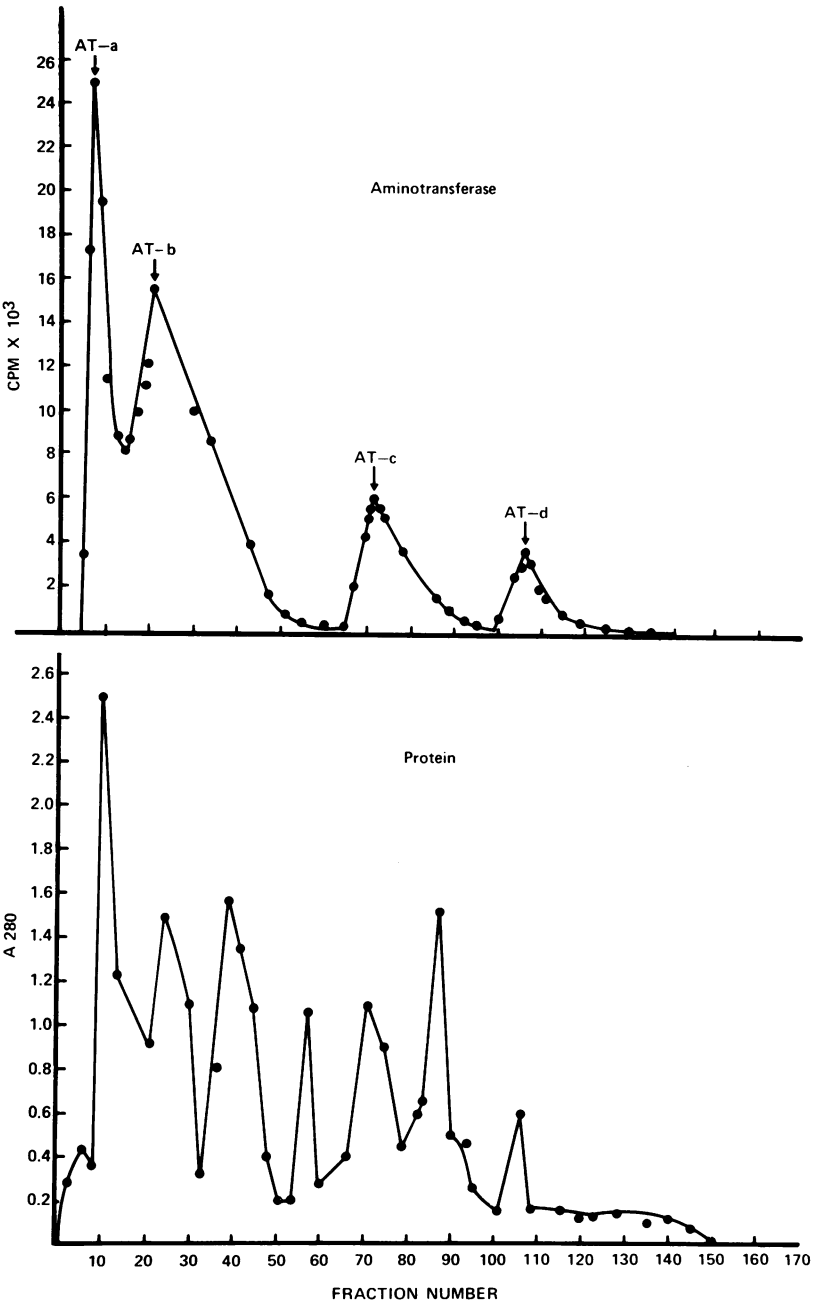

FIG. 9. *Resolution of four species of prephenate aminotransferase after DEAE-cellulose chromatography, eluting the sample of crude extract applied with a linear gradient of 0 to 0.3 M KCl as described in the text. The first 45 fractions represent the 100-ml wash eluate before salt gradient elution. The peak fraction of aminotransferase c (AT-c) eluted at 0.10 M KCl, and the peak fraction of AT-d eluted at 0.19 M KCl. The specific activities of the four aminotransferases in peak fractions were (nanomoles per minute per milligram of protein): AT-a (9.5), AT-b (9.0), AT-c (11.8), and AT-d (17.4). The mixed-substrate assay described in the text was used. A280, Absorbance at 280 nm.*

TABLE 2. *Substrate specificities of prephenate aminotransferases (AT) of Euglena spp.*[a]

Keto acid substrate	^{14}C-labeled L-amino acid substrate	Sp act (μmol/min per mg of protein)			
		AT-a	AT-b	AT-c	AT-d
Prephenate	L-Glutamate	1.65	0.39	0.39	1.41
Phenylpyruvate		2.06	0.43	1.41	1.33
4-Hydroxyphenylpyruvate		2.03	0.56	0.89	1.20
Prephenate	L-Aspartate	0.24	0.14	0	0
Phenylpyruvate		0.98	0	0	0
4-Hydroxyphenylpyruvate		1.10	0.06	0	0
2-Ketoglutarate		1.43	0.25	0.04	0
Prephenate	L-Leucine	0.20	0	0.10	0
Phenylpyruvate		0.98	0.07	0.23	0
4-Hydroxyphenylpyruvate		0.23	0.06	0.10	0
2-Ketoglutarate		5.59	0.67	4.12	0
Prephenate	L-Phenylalanine	0.43	0	0	0
4-Hydroxyphenylpyruvate		0.57	0	0	0
2-Ketoglutarate		0.57	0	0	0
Prephenate	L-Tyrosine	0.81	0	0	0
Phenylpyruvate		1.13	0	0	0
2-Ketoglutarate		0.89	0	0	0

[a] The aromatic aminotransferases were partially purified as described in the legend to Fig. 9.

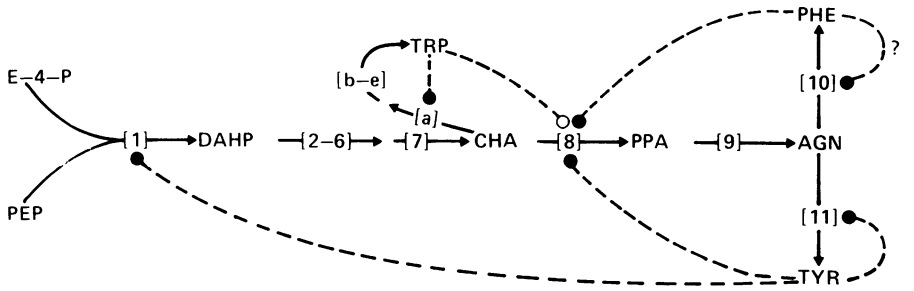

FIG. 10. *Regulation of aromatic amino acid biosynthesis in E. gracilis. Regulation symbols: (-- ● --) feedback inhibition; (-- ○ --) activation. The enzymes corresponding to the numbers shown are: [1] DAHP synthase; [2-6] a multifunctional protein catalyzing the reactions of dehydroquinate synthase, dehydroquinase, dehydroshikimate reductase, shikimate kinase, and 3-enolpyruvylshikimate 5-phosphate synthase; [7] chorismate synthetase; [8] chorismate mutase; [9] prephenate aminotransferase; [10] arogenate dehydratase; [11] arogenate dehydrogenase. Within the tryptophan branchlet, [a] denotes anthranilate synthase and [b-e] denotes the remaining four enzyme steps of tryptophan biosynthesis. Abbreviations: E-4-P, erythrose-4-phosphate; PEP, phosphoenolpyruvate; CHA, chorismate; PPA, prephenate; AGN, L-arogenate; PHE, L-phenylalanine; TYR, L-tyrosine; TRP, L-tryptophan.*

the assay of phenylalanine formation in the presence of added phenylalanine).

E. gracilis exhibits two large multifunctional proteins, one spanning five contiguous steps of the common shikimate pathway and the other spanning the last four steps of tryptophan synthesis. In each case the multifunctional protein follows a separate allosteric protein, i.e., DAHP synthase or anthranilate synthase. Although arogenate dehydratase and arogenate dehydrogenase coeluted from DEAE-cellulose, it seems likely that they will prove to be independent proteins. The remaining proteins beyond chorismate mutase were all separated from one another after DEAE-cellulose chromatography.

Evolutionary relationship with fungi? A variety of enzymological results have encouraged speculations that euglenoids and fungi bear

an evolutionary relationship to one another. For example, *N. crassa* and other fungi possess the same multifunctional protein within the shikimate pathway (1) as is found in *Euglena* spp. (7, 26). These enzymes are separated proteins in a variety of bacteria (3) and other photosynthetic organisms (2). Second, tryptophan synthase of *E. gracilis* was found to resemble the fungal enzyme (24), differing from a large number of other organisms (5, 11, 32, 38). Finally, euglenoids use the L-α-aminoadipate pathway for lysine biosynthesis similar to higher and some lower fungi (34, 35), in contrast to other organisms which depend upon the *meso*-α-diaminopimelate pathway.

The enzymological diversity in phenylalanine and tyrosine biosynthesis has proven enormously successful in identifying taxa within the family *Pseudomonadaceae* (8). Each enzyme system has been suggested to be a highly conserved unit derived from an ancient evolutionary event (22). To date the data available from fungi are too sparse and scattered to identify patterns that distribute along taxonomic and evolutionary lines. Thus far, we know that *N. crassa* lacks the arogenate route to either phenylalanine or tyrosine (23). On the other hand, the ascomycete *Hansenula henricii* utilizes the arogenate route for tyrosine biosynthesis and the phenylpyruvate route for phenylalanine biosynthesis (4). If there should prove to be a trend for utilization of arogenate routes more by lower fungi than by higher fungi, this would imply a relationship of euglenoids to those lower fungi which use the L-α-aminoadipate pathway of lysine biosynthesis, e.g., members of the order *Blastocladiales*.

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