JOURNAL OF BACTERIOLOGY, Sept. 1976, p. 1085-1097
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Vol. 127, No. 3
Printed in U.S.A.

Constitutive and Repressible Enzymes of the Common Pathway of Aromatic Biosynthesis in *Escherichia coli* K-12: Regulation of Enzyme Synthesis at Different Growth Rates

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Received for publication 3 March 1976

Synthesis of five of the enzymes of the common pathway of aromatic biosynthesis has been shown to be unaffected by either the aromatic amino acids—the product of the first reaction (3-deoxy-D-arabinoheptulosonic acid-7-phosphate) or the product of the last reaction (chorismate)—or by the state of regulator gene loci *trpR* and *tyrR*. On the other hand, the rate of synthesis of these enzymes, and of several other enzymes for which repression control was inactive because of mutations in relevant regulator genes, was found to change with growth rate. These changes were found to correlate at faster growth rates than those observed in glucose minimal medium with the alterations in the relative frequencies of the corresponding structural genes which occur at these growth rates. It was also found that when wild-type cells were grown at these faster growth rates in medium lacking the aromatic amino acids, complete derepression of the tyrosine-inhibitable 3-deoxy-D-arabinoheptulosonic acid-7-phosphate synthetase occurred, in strong contrast to the situation when wild-type cells are grown in glucose minimal medium.

The series of reactions in the common pathway of aromatic biosynthesis by which D-erythrose 4-phosphate and phosphoenolpyruvate are converted to chorismate are shown in Fig. 1. Chorismate is the branch point for pathways diverging to tyrosine, phenylalanine, tryptophan, ubiquinone, menaquinone, folic acid, and enterochelin (14, 16, 30). The first reaction of the common pathway is controlled in *Escherichia coli* by each of the major end products, tyrosine, phenylalanine, and tryptophan, and by both feedback inhibition and repression of the three isofunctional enzymes that are involved (30). The only reaction of the common pathway for which there is evidence of some form of specific control is the shikimate kinase (EC 2.7.1.71) reaction, a step which is carried out by two isofunctional enzymes (1; B. K. Ely and J. Pittard, Proc. Aust. Biochem. Soc. 8:56, 1975).

Structural genes for all the enzymes of the pathway except shikimate kinase have been located (36); they are scattered at different locations on the chromosome (Fig. 2). Two regulator genes, *trpR* and *tyrR*, have been identified as being involved in control of the repressible enzymes of the common pathway. In the presence of tryptophan, the *trpR* gene product represses the synthesis of 3-deoxy-D-arabinoheptulosonic acid-7-phosphate (DAHP) synthetase (EC 4.1.2.15) Trp (2, 29). The *tyrR* gene product is implicated together with tyrosine in the repression of both DAHP synthetase Tyr and one of the shikimate kinase enzymes (14; B. K. Ely and J. Pittard, Proc. Aust. Biochem. Soc. 8:56, 1975) and with phenylalanine in repression of the synthesis of DAHP synthetase Phe (3, 18).

Reports by Brown (2), Brown and Somerville (3), and Moldoványi and Dénes (23) bear on the regulation of three of the remaining enzymes of the common pathway, 3-dehydroquinate (DHQ) dehydratase (EC 4.2.1.10) and 3-dehydroshikimate (DHS) reductase (EC 1.1.1.25). Brown (2) found that starvation of an aromatic auxotroph for end products gave no significant derepression of DHQ synthetase (as yet not given an EC number); both he and Moldoványi and Dénes (23) found that this starvation also gave no significant derepression of DHQ dehydratase or DHQ reductase. In addition, Brown and Somerville found levels of DHQ synthetase, and DHQ dehydratase in cells of a *tyrR* mutant were found to be the same as in cells of the wild type (3). Gollub et al. (15) have found that in *Salmonella typhimurium* the levels of all the common pathway enzymes except DAHP synthetase re-

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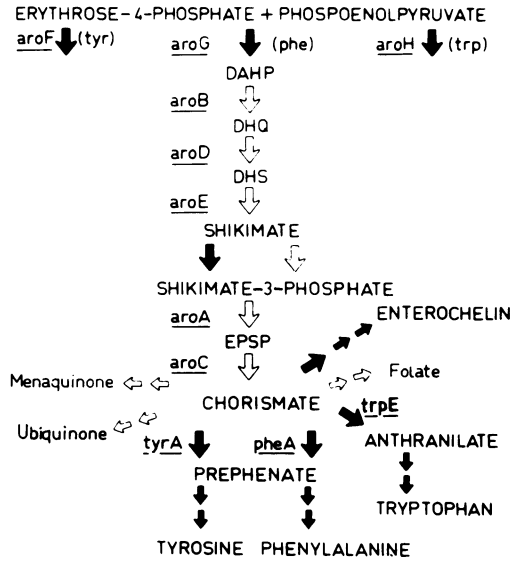

FIG. 1. *An outline of the reactions involved in the synthesis of chorismate from* D-*erythrose 4-phosphate and phosphoenolpyruvate and of the pathways branching from chorismate. The genetic symbols corresponding to each of the known structural genes of the common pathway are included, as well as those for the enzymes involved in the first reactions in the pathways leading to tyrosine, phenylalanine, and tryptophan. Symbols used: large arrows represent the relevant individual enzymes; small arrows represent the remaining terminal pathways; filled arrows represent repressible enzymes; unfilled arrows represent constitutive enzymes. (Formerly a different system to that used here has been employed for numbering the cyclic intermediates of the common pathway.)*

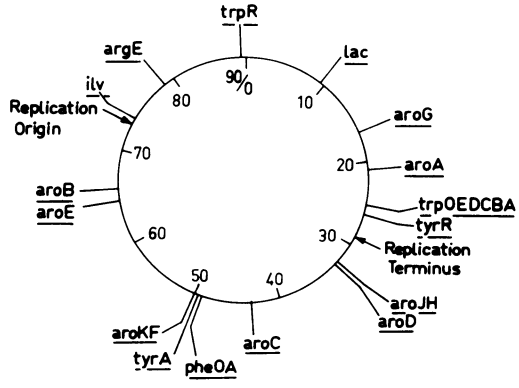

FIG. 2. *Genetic linkage map of the chromosome of E. coli K-12 after Taylor and Trotter (36), with the exception of gene order in the aroF region which is based on unpublished data of D. E. Tribe, H. Wijsman, and S. Im. (The gene-enzyme relationships for most of these genes are given in Fig. 1 and in the footnote to Table 1.) Also shown are the likely origin and terminus of chromosome replication (21).*

main the same whether cells are grown in the presence or absence of excess aromatic amino acids. They also found that growth of cells in enriched medium or starvation of an auxotroph for end products resulted in elevation of the levels of DAHP synthetase, but neither of these procedures was found to affect the levels of DHQ synthetase, DHQ dehydratase, or shikimate kinase.

Fraenkel and Vinopal have emphasized that relatively little is known about the regulation of the synthesis of constitutive enzymes involved in carbohydrate metabolism (11). They commented also that changes in gene frequencies with growth rate may significantly affect the synthesis of these enzymes.

Constitutive synthesis of the tryptophan biosynthetic enzymes in cells which lack specific repression control due to mutation at the *trpR* locus has been studied by Rose and Yanofsky (33), and they have introduced the term "metabolic regulation" to describe the regulation which occurs in the absence of repression control. They observed two patterns of metabolic regulation. (i) At growth rates lower than obtained in glucose minimal medium, rates of enzyme synthesis were directly proportional to cell growth rate. (ii) At higher growth rates, however, rates of enzyme synthesis were significantly depressed relative to the rate obtained with cells grown in glucose minimal medium.

Because *tyrR* is implicated in repression of DAHP synthetase Tyr and shikimate kinase, and *trpR* is implicated in repression of DAHP synthetase Trp, we decided to test if either of these two regulator genes, *tyrR* and *trpR*, is involved further in repression of the synthesis of other enzymes of the common pathway. We also decided to test if the first intermediate of the pathway, DAHP, is involved in induction of these enzymes, or if the last intermediate, chorismate, is involved in repression.

Since the results we obtained suggest that the synthesis of these enzymes is not subject to specific regulation, further studies were then carried out to examine the nature of metabolic regulation affecting these enzymes and to assess the effects of changes in relative gene frequencies on enzyme synthesis.

(A preliminary report of these findings has been made [D. E. Tribe, H. Camakaris, and J. Pittard, Proc. Aust. Biochem. Soc. 8:57, 1975].)

MATERIALS AND METHODS

Organisms. Strains used in this work were all derivatives of *E. coli* K-12 and are described in Table 1.

Growth media. The minimal medium used was medium 56 described by Monod et al. (24), supple-

TABLE 1. *Description of strains of E. coli K-12*

Strain designation	Characteristics[a]	Source or reference
W3110	Prototrophic	C. Yanofsky
AT2594	*argR38*	CGSC[b] (B. Bachmann)
JP62	*aroF363 aroG365 aroH⁺ trpR363*	(reference 29)
JP66	*aroF363 aroG365 aroH371 trpR⁺* (DAHP synthetase Trp resistant to feedback inhibition)	(reference 29)
JP2167	*aroF363*, deletion (*nadA aroG gal*) *aroH367*	This paper
JP2228	As for JP2167, but *tyrR366*	From JP2167 by P1*kc* transduction
JP2240	As for JP2228, but *aroF394 pheA1 tyrA4 aroB351* (DAHP synthetase Tyr resistant to feedback inhibition)	From JP2228, involving several intermediate strains
JP2241	As for JP2240, but *aroB⁺ trpE401*	From JP2228, involving several intermediate strains
JP2245	As for JP2240, but *aroB⁺ trpR363 trp382* (anthranilate synthetase resistant to feedback inhibition)	From JP2228 involving several intermediate strains
JP2247	*aroG397 aroA2* (DAHP synthetase Phe resistant to feedback inhibition)	This paper
JP2310	*tyrR370 trp⁺ trpR⁺* (the phenotypic effects of *tyrR370* are suppressible by amber suppressors)	From JP2410 (described in reference 4) by P1*kc* transduction
JP2311	*tyrR⁺ trp⁺ trpR⁺*	From JP2140 (reference 4) by P1*kc* transduction
JP2407	*tyrR⁺ trp⁺ trpR⁺*	This strain has a genetic background similar to that of JP2311 and was derived from LS446a (reference 4)

[a] *argR* is a regulatory gene controlling the synthesis of enzymes of arginine biosynthesis. *aroF* is the structural gene for DAHP synthetase Tyr. *aroG* is the structural gene for DAHP synthetase Phe. *aroH* is the structural gene for DAHP synthetase Trp. *trpR* is a regulator gene controlling the formation of DAHP synthetase Trp and also the enzymes of the terminal pathway of tryptophan biosynthesis. *nadA* is a structural gene for an enzyme concerned with the synthesis of nicotinic acid. *gal* a gene concerned with ability to utilize galactose. *pheA* is the structural gene for chorismate mutase P-prephenate dehydratase. *tyrA* is the structural gene for chorismate mutase T-prephenate dehydrogenase. *aroB* is the structural gene for DHQ synthetase. *trpE* is the structural gene for anthranilate synthase. *aroA* is the structural gene for EPSP synthetase.

[b] CGSC, Coli Genetic Stock Center, Yale University School of Medicine, New Haven, Conn.

mented with 0.5% glucose or other carbon sources as indicated, thiamine, and appropriate growth factors. Medium 17AA is glucose-medium 56 enriched as described by Novick and Maas (27) for their medium AF, with the modification that phenylalanine, tyrosine, and tryptophan were omitted and that L-arginine was added to give a final concentration of 8×10^{-4} M. Medium 20AA was medium 17AA with 10^{-3} M L-tyrosine and 5×10^{-4} M L-tryptophan added. These concentrations of aromatic amino acids were also used whenever a supplement of aromatic amino acids was made to minimal medium used for growth of cells prior to enzyme assay. Glucose broth is glucose minimal medium with 1% Oxoid tryptone and 0.5% Oxoid yeast extract powder added. Nutrient broth was Oxoid nutrient broth no. 2.

Buffers. The sodium phosphate, tris(hydroxymethyl)aminomethane (Tris) and sodium 5,5-diethylbarbiturate (veronal) buffers used were prepared by the method of Dawson and Elliott (10).

Materials. Unless otherwise specified, the chemicals used were obtained commercially and were not further purified. The preparation of D-erythrose 4-phosphate and phosphoenolpyruvate was as used previously (4). DAHP was prepared by the procedure of Hoch and Nester (17), with the exception that partially purified DAHP synthetase Tyr from *E. coli* was used as a source of enzyme activity. Shikimate 3-phosphate (barium salt) was prepared from the culture fluid of *E. coli* strain JP2247 after growth in glucose minimal medium containing 7×10^{-5} M L-phenylalanine, 7×10^{-5} M L-tyrosine, and 2.5×10^{-5} M L-tryptophan, using the method of Knowles and Sprinson (20). The purified barium salt of shikimate 3-phosphate was converted to the potassium salt with a twofold molar excess of potassium sulfate and used in the enzymatic synthesis of 5-enolpyruvylshikimate 3-phosphate (EPSP) by the method of Knowles et al. (19); *E. coli* strain W3110 was used as a source of EPSP synthetase (as yet not given an EC number) activity in this procedure.

Chorismic acid was prepared by the method of Gibson (13).

Growth of cells for assay of enzyme activity. For each cell extract prepared, a 200-ml culture was grown in a rotary shaker at 37°C using as an inocu-

lum an unwashed 14-h culture in the same medium. Increases in turbidity were followed using a Klett-Summerson photoelectric colorimeter with a no. 54 filter. After the cells had undergone at least four mass doublings, growth was terminated in midexponential phase by transfer of flasks to an ice bath.

Preparation of cell extracts for enzyme assay. After being washed twice in 0.9% NaCl, the cells from each 200-ml culture were suspended in 5 ml of buffer. This buffer was 0.1 M sodium phosphate, pH 7.0, containing 10^{-4} M ethylenediaminetetraacetic acid in the case of extracts prepared for assay of DAHP synthetase, DHQ synthetase, DHQ dehydratase, or DHS reductase. For assay of other enzyme activities an extract was prepared through suspension of cells from a separate but otherwise identical 200-ml culture in a different buffer. According to which enzyme assays were carried out, the buffers were: 0.04 M veronal, pH 9.0, prior to assay of shikimate kinase; 0.01 M Tris, pH 7.6, before the EPSP synthetase assay; 0.01 M Tris, pH 7.6, containing 10^{-4} M reduced glutathione, before chorismate synthetase assay; and 0.1 M sodium phosphate, pH 7.0, before assay of acetylornithine deacetylase (EC 3.5.1.16).

Cell breakage was then achieved using either an MSE 500W ultrasonic disintegrator (Measuring and Scientific Equipment Ltd.) or a French press. Cobaltous chloride was added immediately after cell disruption to give a final concentration of 10^{-3} M, in extracts which were to be assayed for DAHP synthetase or DHQ synthetase. All extracts were clarified by centrifugation and always assayed for enzyme activity within 1 h of preparation, with the exception of extracts assayed for EPSP synthetase, which were subjected to dialysis overnight as described by Gollub et al. (15).

Enzyme assays. These were all carried out at 37°C under conditions of linearity with respect to time and amount of enzyme. The unit of activity, which differs from that used in previous reports from this laboratory, is the amount of enzyme converting 1 μmol of substrate or producing 1 μmol of product per min at 37°C. Specific activities are given as milliunits of enzyme activity per milligram of protein.

DAHP synthetase was assayed as described previously (4). DAHP synthetase Phe was determined as the amount of total activity inhibitable by 2.5×10^{-4} M L-phenylalanine and, similarly, DAHP synthetase Tyr as the amount of total activity inhibitable by 2.5×10^{-4} M L-tyrosine.

DHQ synthetase and EPSP synthetase were assayed by the methods used by Gollub et al. (15).

DHS reductase. The reaction was started by adding 6 μmol of DHS to a cuvette containing 300 μmol of Tris buffer, pH 8.0, and 1.0 μmol of reduced nicotinamide adenine dinucleotide phosphate and enzyme in a final volume of 3.0 ml. After rapid mixing, the change in absorbance by reduced nicotinamide adenine dinucleotide phosphate at 340 nm was measured for 2 to 3 min in a Unicam SP 500 series 2 spectrophotometer coupled to a Sargent model SRL recorder.

Assay of shikimate kinase. The reaction was started by addition of cell extracts to 1 μmol of shikimic acid, 4 μmol of $MgCl_2$, 10 μmol of NaF, and 25 μmol of veronal buffer, pH 9.0, in a final volume of 1.0 ml; 100 μl was withdrawn into 3.0 ml of water at 0°C at 0, 5, 10, 15, 20, and 25 min. These diluted samples were then assayed for shikimate as described by Gationde and Gordon (12). Enzyme activities were based on the initial rate of reaction.

Chorismate synthetase was assayed by the method of Morell et al. (25). To each assay an excess (of at least 500-fold in enzyme activity) of highly purified chorismate mutase P (EC 5.4.99.5)-prephenate dehydratase (EC 4.2.1.51), which was the kind gift of B. Davidson, was added.

The method of Pittard et al. (29) was used for assay of anthranilate synthase, and the method of Vogel and McLellan (37) was used for assay of acetylornithine deacetylase.

Protein estimation. Protein was estimated by the method of Lowry et al. (22) using bovine serum albumin as a standard.

Assay of culture fluids. DAHP and DAH, the dephosphorylated form of DAHP, were assayed as described previously (29). Chorismate was assayed as follows. A 45-ml sample was acidified with 5 ml of 1 M HCl and extracted repeatedly with ether. The ether extracts were combined (total volume, 20 ml) and concentrated by rotary evaporation at 20°C. When the volume had reached about 5 ml, 3 ml of 0.1 M phosphate buffer was added, and the remainder of the ether was evaporated. Samples were then taken for conversion to anthranilic acid using reaction conditions of the anthranilate synthase assay. Strain JP2245 was used as a source of anthranilate synthase activity.

Relation of relative gene frequency to culture growth rate. For calculation of relative gene frequency per mass of deoxyribonucleic acid (DNA) ($\bar{F}_G$) from the growth rate of a culture in doublings per hour (μ) and the relative distance of a gene from the origin of replication (x), the following equation was used:

$$F_G = \frac{c\ln 2}{60} \times \mu \times 2^{c\cdot\frac{\mu}{60}\cdot(1-x)} \times \frac{1}{2^{c\cdot\frac{\mu}{60}} - 1} \quad (1)$$

A derivation of this equation is given by Collins and Pritchard (7). The equation is valid for a culture in which: (i) the cells undergo chromosomal replication by the Cooper-Helmstetter model (8); (ii) each cell in the culture has the same interdivision time; and (iii) the cells of the culture are in a steady state of growth with a cell age distribution as described by Powell (31). For our calculations we assume that the transit time for the chromosome replication fork (parameter c of equation 1) does not vary with growth rate (8) and that a value of $c = 45$ is appropriate for strains of *E. coli* K-12 grown in batch culture (7 and 8; see Discussion).

Relation rates of protein, aromatic amino acid, and enzyme synthesis to growth rate. The fact that each cellular component of a culture in steady-state growth increases with the same growth rate μ was

used to derive four further equations (2 through 5) which were used in calculation of rates of cellular reactions.

Equation 2 was used to calculate the rate of protein synthesis per mass of DNA (p) from the growth rate and the ratio of protein (P) to DNA (D) in a culture. It was assumed that P/D does not change with growth rate (34, 35).

$$p = \frac{\ln 2}{60} \times \frac{P}{D} \times \mu \quad (2)$$

Equation 3 was used to calculate the net rate of incorporation of aromatic amino acids into protein (A), per mass of protein $\{[d(A)/dt] \times [1/P]\}$ from growth rate and published values for the aromatic amino acid (32) and protein content (34) of *E. coli*.

$$\frac{d(A)}{dt} \times \frac{1}{P} = \frac{\ln 2}{60} \times \frac{A}{P} \times \mu \quad (3)$$

Equation 4 was used to calculate the rate of enzyme synthesis per mass of DNA (q) from growth rate and enzyme specific activity (S) data. This equation is valid under conditions where the rate of enzyme breakdown is insignificant compared with the rate of synthesis, and enzyme activities found in cell extracts are proportional to the number of molecules present.

$$q = \frac{\ln 2}{60} \times \frac{P}{D} \times S \times \mu \quad (4)$$

From q values, the rate of enzyme synthesis per gene (q_g) was calculated as follows:

$$q_g = \frac{q}{\text{number of genes per mg of DNA}} = \frac{q}{\overline{F}_G} \times 4.22 \times 10^{-2} \text{ mg} \quad (5)$$

RESULTS

Effect of mutations in the regulator genes *tyrR* and *trpR* on the levels of common pathway enzymes. (i) ***tyrR***. Strain JP2310 possesses an amber-suppressible mutation, *tyrR370*, but does not carry an active amber suppressor gene. It is assumed that in this strain the *tyrR* gene product is sufficiently altered to render it totally inactive. The high levels of DAHP synthetase Tyr, DAHP synthetase Phe, and shikimate kinase found in cells of this strain grown both in minimal medium and in the presence of tyrosine support this assumption (4; B. K. Ely and J. Pittard, Proc. Aust. Biochem. Soc. 8:56, 1975). Strain JP2311 is the *tyrR*$^+$ control strain in which the wild-type *tyrR* gene product is produced. Neither the presence nor absence of mutant *tyrR370* allele or a supplement of aromatic amino acids to the growth medium makes any significant alteration to the level of common pathway enzymes (Table 2). JP2228 is another *tyrR*$^-$ strain, and JP2167 is its *tyrR*$^+$ parent. (The *tyrR366* allele of JP2228 is not amber suppressible and arose independently of the *tyrR370* mutation.) The levels of common pathway enzyme present in strain JP2167 and JP2228 as given in Table 2 are not significantly different from those of *tyrR*$^+$ strain JP2311 and support the results obtained with the amber *tyrR*$^-$ mutant.

(ii) ***trpR***. The strain JP2407 is a strain with a mutation in the *trpR* gene which causes the tryptophan biosynthetic enzymes to be produced constitutively (D. E. Tribe, unpublished observations). The level of common pathway enzymes are the same in this strain as in JP2311, which is wild type at the *trpR* locus. Furthermore, addition of aromatic amino acids to the medium used for growth of cells has no effect on enzyme levels in either strain (Table 2).

Effect of common pathway intermediates on the synthesis of common pathway enzymes. The finding that chorismate is unable to replace the 4-aminobenzoate requirement of a multiple aromatic auxotroph (13) suggests that chorismate is not effectively transported into the *E. coli* cells. (4-Aminobenzoate is an intermediate on the pathway between chorismate and folic acid.) We assume there may also be a barrier to entry of DAHP, a concept which is consistent with failure of early feeding studies with auxotrophic mutants to reveal an intermediate prior to DHQ in the common pathway (for a summary of these early studies see reference 16). Consequently, to test whether either of these compounds (DAHP or chorismate) acts to either induce or repress the synthesis of common pathway enzymes we chose to use strains which accumulate these compounds endogenously rather than to study the effect of adding the compounds to growth media. One such strain is JP2240. It contains only a single functional DAHP synthetase, DAHP synthetase Tyr, and because of a mutation *aroF394* this enzyme is resistant to feedback inhibition; since strain JP2240 carries the *tyrR366* allele, the synthesis of the enzyme is not subject to repression. Since JP2240 also contains a mutation in the gene for DHQ synthetase it accumulates DAH(P) during growth. The supernatant fluid remaining after harvesting cells of JP2240 for enzyme assay contained 0.5 mM DAH(P).

This strain grows slowly in media supplemented with its aromatic amino acid and vitamin requirements (μ = 0.4 to 0.46 doubling/h compared with 0.8 to 1.0 doubling/h for the

TABLE 2. *Specific activity of enzymes of the common pathway in regulatory mutants*

Strain	Relevant details of genotype and phenotype	Growth medium[a]	Sp act (mU/mg of protein)				
			DHQ synthetase	DHQ dehydratase	DHS reductase	EPSP synthetase	Chorismate synthetase
JP2311	*tyrR*$^+$ *trpR*$^+$	MM	27	55, 55[b]	60	70, 120[c]	9.5
		MMA	23	70, 75	65	130, 115	9.5
JP2310	*tyrR370* (amber)	MM	25	70, 70, 70	55, 60, 65	100, 95, 110	9.5
		MMA	24	65, 55, 85	60, 60	110	11.0
JP2407	*trpR*$^-$	MM	20	75	60	100	10.0
		MMA	20	75	60	85	10.0
JP2167	*aroF*$^-$ *G*$^-$ *H*$^-$ *tyrR*$^+$[d]	Iron citrate	ND[e]	ND	55	90, 50	10.5
JP2228	*aroF*$^-$ *G*$^-$ *H*$^-$ *tyrR366*[d]	Iron citrate	22	55, 50	55	70	10.5
JP2240	*aroF394* (feedback resistant) *tyrR366 aroB*$^-$, accumulates DAHP	Iron citrate	ND	65	50	80	8.0
JP2241	*aroF394* (feedback resistant) *tyrR366 pheA*$^-$ *trpE*$^-$, accumulates chorismate	Iron citrate	23	65, 60	50	95	9.0

[a] Abbreviations: MM, Glucose minimal medium; MMA, MM plus 10^{-3} M L-tyrosine, 10^{-3} M L-phenylalanine, and 5×10^{-4} M L-tryptophan. Iron citrate consisted of MM plus 200 μM $FeCl_3$ and 10 mM citrate, which were added to ensure that the enzymes of enterochelin biosynthesis were repressed (38).

[b] These are the results of assays carried out on separate extracts.

[c] The variation in the data for EPSP activity represents differences in enzyme activity found in different extracts; the reasons for the variation were not further investigated.

[d] Strains JP2167 and JP2228 have a genetic background similar to that of JP2240 and JP2241, and assays were carried out on these strains to provide a reference level to the results with JP2240 and JP2241.

[e] ND, Not determined.

control strain JP2228), and its growth rate is not significantly increased by supplementation of the medium with shikimate (D. Tribe, unpublished observations). It is assumed that this slow growth is due to the accumulation of toxic concentrations of intracellular DAHP.

An examination of Table 2 shows that there are no significant differences between the levels of common pathway enzymes involved in conversion of DAHP to chorismate in strains JP2240 and JP2228. Therefore, we conclude that DAHP does not directly induce synthesis of enzymes occurring later in the pathway.

Chorismate. The last intermediate in the common pathway is chorismic acid. A possible role for chorismate in repression of the synthesis of common pathway enzymes was tested in strain JP2241. This strain, in addition to being both feedback resistant and having derepressed levels of DAHP synthetase Tyr, is blocked in the three major pathways utilizing chorismic acid, namely, chorismate mutase P-prephenate dehydratase, chorismate mutase T (EC 5.4.99.5)-prephenate dehydrogenase (EC 1.3.1.12), and anthranilate synthase. Since the rate of synthesis of enterochelin may in certain circumstances form a significant fraction of the total products of the common pathway (D. E. Tribe, unpublished observations) this strain was grown in the presence of 200 μM $FeCl_3$ plus 10 mM citrate to repress synthesis of the enzymes of the enterochelin pathway and limit conversion of chorismate to enterochelin (38). The supernatant fluid obtained after harvesting cells of JP2241 contained 0.12 mM chorismate.

Inspection of Table 2 reveals that the levels of common pathway enzymes are not affected by chorismate accumulation.

Variation in the rate of synthesis of common pathway enzymes with growth rate. In the experiments described so far, all the strains (with the exception noted of strain JP2240) grew at similar growth rates, and comparisons of specific activity values were made to interpret the experiments.

Specific activity data are useful, since they give the ratio of rate of enzyme synthesis to the total rate of protein synthesis. However, when cells are grown at different growth rates the total rate of protein synthesis per mass of DNA (p) varies in direct proportion to μ (Materials and Methods, equation 2), and this variation complicates interpretation of specific activity data. Consequently, in the experiments reported in the remaining sections, in which cells were grown at different growth rates, we will follow an extension of the approach of Rose and Yanofsky (33) and calculate rates of enzyme synthesis per mass of DNA (q), or per gene (q_g), from experimental values of μ and specific activity, using either

equation 4 or 5 of Materials and Methods. Furthermore, to allow assessment of the degree of derepression of the repressible enzymes in wild-type cells these rates of synthesis are expressed as a percentage of the rate of synthesis found with *tyrR* amber mutant.

We wish to focus attention on the question of whether constitutive enzyme synthesis is regulated coordinately with total rate of protein synthesis. Figure 3 shows several ways in which q may be expected to vary with μ, based on calculations using equations 1, 2, 4, and 5 of Materials and Methods. The line for "constant specific activity" illustrates that coordinate variation of q, p, and μ results if the same enzyme levels are present when cells are grown at different μ values. Curves A, B, and C show the variation in q for two different models of constitutive enzyme synthesis, the details of which are given in the legend to the figure. Model I assumes that for values of μ less than 1 doubling/h, q varies coordinately with μ but that above this growth rate q is constant (see curve A). Model II, however, takes into consideration the change in relative gene frequencies, $\bar{F}_g$, which occurs as a consequence of the presence of multiple chromosome replication forks at higher growth rates. For this model, q_g is taken to vary coordinately with μ below a growth rate of 1 doubling/h and to be constant above the growth rate (see curves B and C). Curve B is for a gene located near the origin of chromosome replication, and curve C is for a gene located near the terminus of replication; taken together, they illustrate the maximum extent to which changes in $\bar{F}_G$ are expected to affect q.

For both models, q varies in a closely coordinate fashion with μ and p below growth rates of 1 doubling/h. Above this growth rate, q does not vary coordinately with μ for either model, and for model II the variation in q depends on the position of the structural gene on the chromosome.

In Fig. 4 experimental results for the variation in q of four common pathway enzymes are shown for cells grown at different growth rates obtained by adding different carbon sources (glucose, gluconate, xylose, lactose, maltose, lactate, glycerol, or succinate) to minimal medium and by using glucose broth or minimal medium enriched with Difco Casamino Acids (0.5%). Growth rates ranged from less than 0.1 doubling/h in succinate minimal medium to near 1.8 doublings/h in glucose broth.

The data for the DAHP synthetases were obtained with cells of strain JP2310 in which (because of a mutation in the *tyrR* locus) both

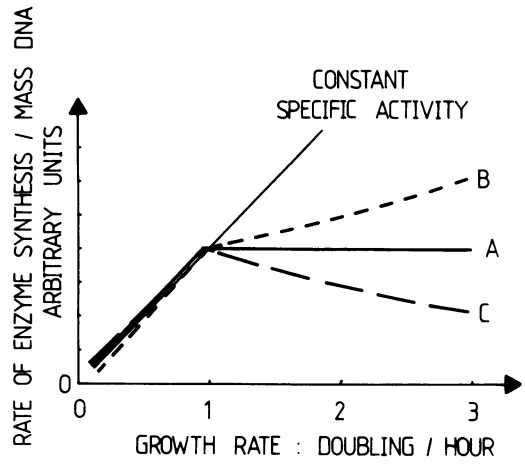

FIG. 3. *The variation in rate of enzyme synthesis per mass of DNA (q) for various models of constitutive enzyme synthesis. The "constant specific activity" line shows the variation of q if enzyme levels are the same at all growth rates, based on equation 4 of Materials and Methods. Model I is illustrated by curve A and assumes specific activity is constant below 1 doubling/h and that q is constant above this growth rate. Model II, which is illustrated by curves B and C, is derived from the values of curve A by multiplying by the relative gene frequency given by equation 1 of Materials and Methods and by normalizing so that curve A is intersected at a growth rate of 1 doubling/h. Curve B is for a gene at the origin of replication and curve C is for a gene at the terminus. They both illustrate the variation of q if the rate of enzyme synthesis per gene (q_g) varies coordinately with growth rate below 1 doubling/h, but is constant above this growth rate.*

isoenzymes examined are produced constitutively. For the other two enzymes, DHQ dehydratase and DHS reductase, similar results were obtained with JP2310 and with W3110, and the data represent the pooled results from both of these strains. In each case the rate of enzyme synthesis varies considerably with the increase in growth rate up to a growth rate of approximately 0.8 doubling/h. At faster growth rates the results differ from enzyme to enzyme. In the case of DHS reductase, the rate of enzyme synthesis continues to increase, as does that of DAHP synthetase Tyr. DHQ dehydratase and DAHP synthetase Phe, on the other hand, show decreased rates of enzyme synthesis at the high growth rates. In considering these results it was interesting to note (Fig. 2) that the structural gene for DHS reductase at min 64 on the map is close to the origin of chromosomal replication, whereas the structural gene for DHQ dehydratase is close to the terminus of replication. In a similar fashion, whereas the structural gene for DAHP

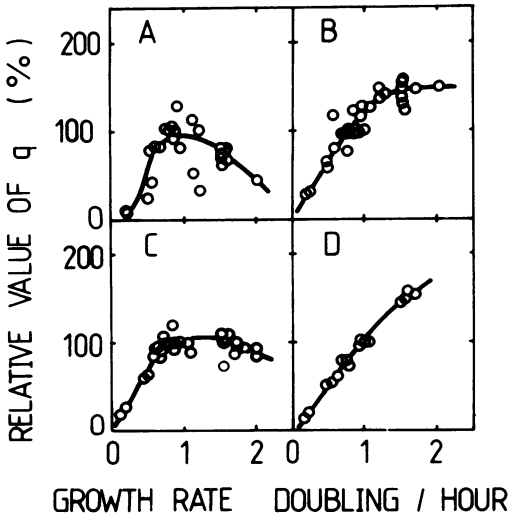

FIG. 4. *The rate of synthesis of four common pathway enzymes in cells grown at different growth rates. Values of rate of enzyme synthesis per mass of DNA (q) were calculated from growth rate and specific activity data using equation 4 of Materials and Methods; to facilitate comparisons the relative values expressed as a percentage of the rate of synthesis in glucose minimal medium are plotted. (A) Relative rate of synthesis of DAHP synthetase Phe in cells of strain JP2310. (B) Relative rate of synthesis of DAHP synthetase Tyr in cells of strain JP2310. (C) Relative rate of synthesis of DHQ dehydratase. (D) Relative rate of synthesis of DHS reductase. In (C) and (D) the data represent the pooled results of experiments carried out with strains JP2310 and W3110. The fit of the lines drawn through the experimental points was assessed visually.*

synthetase Tyr at min 51 is situated approximately midway between origin and terminus, the structural gene for DAHP synthetase Phe at min 17 is much closer to the terminus. Relative gene frequency changes might have been expected to influence the rates of enzyme synthesis in the direction observed, although calculations of the extent of these effects using equation 1 of Materials and Methods (which are illustrated by curves B and C of Fig. 3) indicate that this offers only a partial explanation for the results.

To examine the effects of changes in $\bar{F}_G$ in more detail, we then examined a wider range of enzyme activities, and specific activities were used to calculate q values at only two growth rates. The results of this experiment are shown in Fig. 5.

Cells were grown in a rich medium, which was either glucose broth or nutrient broth, and in glucose minimal medium and assayed for enzyme activity, and the ratio of q in rich medium to q in minimal medium was calculated; the experimental values for this ratio have been plotted using as a horizontal axis

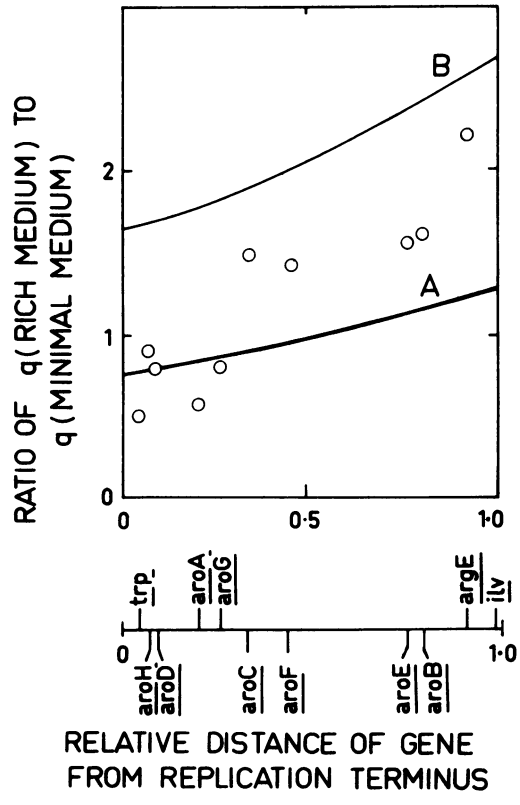

FIG. 5. *Correlation of the changes in rate of enzyme synthesis in enriched media with the location of structural gene in relation to the origin and terminus of chromosomal replication. Cells were grown in glucose minimal medium and in a rich medium (either glucose broth or nutrient broth), and enzyme assays were carried out. Rates of enzyme synthesis per mass of DNA (q) were then calculated as described in Fig. 4, and the ratio of q (rich medium) to q (glucose minimal medium) was plotted against the relative distance of the corresponding gene from the terminus of replication. The structural genes which correspond to each of the experimental points are indicated on a separate axis below the horizontal axis of the graph. Also shown are: (A) the expected result if rates of enzyme synthesis per gene (q_s) were the same in rich and minimal media and (B) the expected result if q_s increases coordinately with growth rate, both calculated using the gene frequencies given by equation 1 of Materials and Methods. The growth rates in rich medium were close to 1.7 doublings/h, and in minimal medium, 0.8 doublings/h. In most cases, the values are the average of several separate experiments. For further details, see text.*

the relative distance of the structural gene from the terminus of replication. In the instances where the enzyme activity examined is normally repressible, cells of an appropriate constitutive mutant were used, the strains being the *tyrR* regulator mutant JP2310 in the case of DAHP synthetases Tyr and Phe, the *trpR* mutant JP62 in the case of DAHP synthetase Trp and anthranilate synthase, and the *argR* mutant AT2594 in the case of acetylornithine deacetylase. The wild-type strain W3110 was used to examine the behavior of the other enzymes examined, namely DHQ synthetase, DHQ dehydratase, DHS reductase, EPSP synthetase, and chorismate synthetase. The results obtained with JP62 were obtained using nutrient broth as the rich medium (and are included with the kind permission of J. Camakaris), but for all the others glucose broth was used as the rich medium.

Also given in the figure are curves showing: (i) the variation in the ratio of q (rich medium) to q (minimal medium) expected if q_g is the same at both growth rates and (ii) the variation expected in the ratio of q (rich medium) to q (minimal medium) if q_g varies coordinately with p and μ. It can be seen that although the experimental results did not fit either of the curves, there is a very definite relationship between the position of the structural gene relative to the terminus and the extent of the change in rate of enzyme synthesis at the high growth rates. The results also show that for most enzymes the changes in rate of enzyme synthesis per gene at these higher growth rates are proportionally less than the increase in the total rate of protein synthesis.

Effects of growth in enriched medium lacking aromatic amino acids on the levels of enzyme of the common pathway. Since the difference between the rate of synthesis of both DAHP synthetases Tyr and Phe in cells grown in rich medium and the rate of synthesis in cells grown in glucose minimal medium was proportionally less than the change in total rate of protein synthesis, the total DAHP synthetase activity per milligram of cell protein was lower in extracts prepared from cells grown in rich medium than in extracts of cells grown in glucose minimal medium. If, however, the aromatic amino acids are absent from an enriched medium, equation 3 (Materials and Methods) shows that the rate of incorporation of aromatic amino acids into protein per milligram of cell protein must be higher in direct proportion to the increased growth rate. These considerations suggested the following experiment. The wild-type strain W3110 was grown in glucose minimal medium, in minimal medium enriched with a mixture of the seventeen nonaromatic amino acids plus vitamins and bases (medium 17AA), and in medium 17AA plus the aromatic amino acids (medium 20AA). The results of enzyme assays on extracts of those cells are shown in Table 3.

In the cells grown in minimal medium or medium 20AA, DAHP synthetase Phe was the major DAHP synthetase isoenzyme present. In contrast, in the cells grown in medium 17AA, the DAHP synthetase Tyr was the major

TABLE 3. *Relation of DAHP synthetase and shikimate kinase synthesis to presence or absence of enrichments to minimal medium*

Strain	Relevant genotype	Growth medium[a]	Growth rate (doublings/h)[b]	Sp act (mU/mg of protein)[c]				Relative rate of enzyme synthesis[d]		
				DAHP synthetase			Shikimate kinase	q_g		q (shikimate kinase)
				Total	Tyr	Phe		DAHP synthetase Tyr	DAHP synthetase Phe	
W3110	Wild type	MM	0.97–0.99	265	70	230	15	15	93	35
W3110	Wild type	17AA	1.5	650	525	110	14.5	170	72	52
W3110	Wild type	20AA	1.7–1.8	48	13.5	40	8.0	5	30	33
JP2310	*tyrR*⁻ (amber)	MM	0.83	990	560	300	48	100	100	100
JP2310	*tyrR*⁻ (amber)	20AA	1.7	565	390	125	30	145	93	110

[a] Abbreviations: MM, Glucose minimal medium; 17AA and 20AA, media 17AA and 20AA, respectively.

[b] Equation 3 of Materials and Methods predicts that the rate of incorporation of aromatic amino acids in protein in cells growing at 0.96 doubling/h is 5.04 nmol/mg of protein per min, and 8.3 nmol/mg of protein per min in cells growing at 1.5 doublings/h.

[c] Each value of specific activity is the average of assays on two or more extracts.

[d] Rates of enzyme synthesis for DAHP synthetase Tyr and Phe per gene, q_g, were calculated from specific activity and growth rate data using equation 5 of Materials and Methods and then expressed as a percentage of the value found with the *tyrR*⁻ strain grown in glucose minimal medium. Rates of enzyme synthesis for shikimate kinase per mass of DNA, q, were calculated as in Fig. 4 and expressed as a percentage of the value found with the *tyrR*⁻ strain grown in glucose minimal medium.

activity. In Table 3, q_d for DAHP synthetases Phe and Tyr has been expressed relative to the value obtained for the amber *tyrR* strain JP2310 grown in minimal medium. These calculations of relative rates of enzyme synthesis per gene in cells grown in medium 17AA and in minimal medium indicate that with the wild-type cells at least 10-fold derepression of synthesis of the tyrosine isoenzyme occurred in medium 17AA. In this medium, the synthesis of this isoenzyme was occurring at a fully derepressed rate. The lower specific activity of DAHP synthetase Phe obtained when cells were grown in medium 17AA instead of minimal medium is consistent with no significant change having occurred in the degree of repression of this enzyme.

Assays of shikimate kinase activity was also carried out. Since the positions of both the shikimate kinase structural genes are not known and the relative contributions of the two kinase enzymes to total enzyme activity were not determined, the results have been expressed as relative q rather than relative q_d values, using the rate found with *tyrR*$^-$ cells grown in minimal medium as 100%. Even though the presence of two forms of shikimate kinase complicates the interpretation of these results, the data show that synthesis of shikimate kinase occurred at a greater rate in medium 17AA than in minimal medium. Derepression of shikimate kinase was not coordinate with the derepression of the synthesis of DAHP synthetase Tyr in that q (shikimate kinase) for cells of the *tyrR*$^+$ strain was only 52% of the *tyrR*$^-$ value, in contrast to the degree of derepression of DAHP synthetase Tyr.

The effect of growth in medium 17AA on enzyme levels of other common pathway enzymes is shown in Table 4. The changes in specific activities in the enriched media show that under conditions in which there is maximal depression of the synthesis of DAHP synthetase Tyr there is no evidence that the synthesis of any of these other enzymes is derepressed.

DISCUSSION

Constitutive enzymes of the common pathway. The synthesis of the enzymes of the common pathway other than those involved in reactions I and V has been shown to be constitutive. No derepression of synthesis of these enzymes was observed under conditions that resulted in complete derepression of DAHP synthetase Tyr (Tables 3 and 4), and the addition of aromatic amino acids to the growth medium had no effect on enzyme levels (Table 2).

Furthermore, the synthesis of this group of enzymes was not affected by mutations in the regulator genes *tyrR* and *trpR* (which have previously been implicated in repression of the synthesis of other enzymes of the pathway), and induction or repression control involving DAHP or chorismate as effectors was shown to be unlikely. These findings lead us to suggest that the synthesis of these enzymes is not subject to specific regulation by the levels of either the aromatic amino acids, DAHP, or chorismate.

Gene frequency changes and metabolic regulation. The results of experiments described in Fig. 4 show that for a range of growth rates from 0.1 to 1.0 doublings/h, the change in rate of enzyme synthesis varies coordinately with growth rate. This change cannot be accounted for by changes in gene frequency, and the results are similar to those described by Rose and Yanofsky (33). These workers have suggested that in this range of growth rates many operons are subject to a nonspecific control which alters gene expression coordinately with the total rate of protein synthesis by regulation of messenger ribonucleic acid transcription frequencies; data on the rate of synthesis of β-galactosidase in cells grown in a chemostat at different growth rates are in accord with the *lac* operon being

TABLE 4. *Relation of specific activities of common pathway enzyme to presence or absence of enrichments to minimal medium*[a]

Growth medium	Growth rate (doublings/h)	Sp act (mU/mg of protein)				
		DHQ synthetase	DHQ dehydratase	DHS reductase	EPSP synthetase	Chorismate synthetase
MM	0.94–0.97	25	80	70	130	10
17AA	1.5–1.8	ND[b]	55	65	95	5.5
20AA	1.7–2.1	ND	37	60	25	6.0

[a] Cells of the wild-type strain W3110 were used. The rate of incorporation of aromatic amino acids into protein in cells growing at 0.96 doubling/h is estimated to be 5.04 nmol/mg of protein per min, and at 1.8 doublings/h the rate is estimated to be 10 nmol/mg of protein per min (see footnotels to Table 3).

[b] ND, Not determined.

affected by such a control at low growth rates (6).

A different pattern of metabolic regulation was found, however, with cells which were grown with μ in the range of 0.8 to 2.0 doublings/h. Generally, with glucose broth or with similar "rich" media which give faster growth rates than those with glucose minimal medium, we found that specific activities for a number of enzymes were depressed relative to the values found in cells grown in glucose minimal medium (Tables 3 and 4; Fig. 4 and 5). As we have commented previously (see Results), this implies a noncoordinate variation of enzyme and total protein synthesis. If the data in Fig. 4 and 5 are examined to see if these changes in enzyme levels at these higher growth rates are consistent with a constant rate of enzyme synthesis per gene, it can be seen that although this hypothesis gives an explanation for the overall pattern of results it does not agree quantitatively with the data. For example, the value given in Fig. 5 for anthranilate synthase synthesis corresponds to q (*trp*) after growth of cells in nutrient broth being 50% of the value obtained with cells grown in glucose minimal medium, a result which is quantitatively similar to that which has been reported previously for the degree of derepression of *trp* operon expression in rich media (33). But if q (*trp*) were constant, our calculations show that in these rich media q (*trp*) should be 80% of the glucose minimal medium value. Furthermore, if a lower value is chosen for the constant c, such as the recent estimate of c = 40 min for strains of *E. coli* K-12 (5), than that (c = 45 min) we have used in these calculations, then the discrepancy between the experimental results and the predicted behavior is even greater.

However, if one assumes that the variation from enzyme to enzyme in the ratio of rates of enzyme synthesis shown in Fig. 5 is caused entirely by some factor having an effect on rate of enzyme synthesis which varies according to the relative distance of the structural gene from the terminus of replication, it is possible to predict gene positions from data on rates of enzyme synthesis. For example, the data on rate of shikimate kinase synthesis given in Table 3 can be taken to predict that the structural gene for the shikimate kinase isoenzyme, whose synthesis is controlled by *tyrR*, is (because of the bidirectional nature of the replication) near either of two positions on the chromosome, corresponding to either min 13 or min 45 on the Taylor and Trotter (36) map. Recent work in this laboratory by B. Ely involving study of shikimate kinase enzyme levels in a series of F′ strains has confirmed a location of this shikimate kinase structural gene near min 13: Ely found a doubling of levels of shikimate kinase in *tyrR* strains diploid for the region min 9 to min 15 but discovered no such effect in strains diploid for the region near min 45 (B. Ely, personal communication).

The *lac* operon (Fig. 2) is located at a similar position but on the opposite limb of the chromosome to the structural gene for DAHP synthetase Tyr at *aroF* in relation to the origin and terminus of replication. Consequently, it is expected that if the *lac* operon is affected by metabolic regulation in the same way as the biosynthetic enzymes we have studied, the rates of β-galactosidase and DAHP synthetase Tyr synthesis should vary in a similar fashion at high growth rates. A recent report by Dalbow and Bremer on metabolic regulation of β-galactoside synthesis in cells induced with isopropylthiogalactoside in the presence of cyclic adenosine 3′,5′-monophosphate provides support for this concept (9).

Further analysis of the relationship between our observed changes in rate of enzyme synthesis and gene position will depend on experimental verification that equation 1 of Materials and Methods makes an accurate prediction of relative gene frequencies for cells grown under these conditions.

Relation of common pathway enzyme levels to synthesis of aromatic amino acids. A published estimate of the intracellular phenylalanine and tyrosine pool size for cells of *E. coli* K-12 growing exponentially in glucose minimal medium suggests that there is 1 μmol of each of these amino acids per g of intracellular water (28).

Even if the effective free pool of these amino acids is 10-fold less than this value, the profile of sensitivity of the respective isoenzymes to feedback inhibition (14) suggests that under these growth conditions the activity of DAHP synthetases Phe and Tyr would be inhibited 90% in vivo. The finding that DAHP synthetase activity in extracts of wild-type cells grown in minimal medium is 52-fold greater than the rate of estimated incorporation of aromatic amino acids into protein in growing cells (Table 3) is consistent with this hypothesis. (The activities recovered in cell extracts are only an approximation to the in vivo activity in the absence of feedback inhibition, but data reported by Pittard et al. [29] show that this approximation is unlikely to be a gross overestimate. They describe strain JP66 [which we also describe in our Table 1], which possesses

only a single DAHP synthetase Trp isoenzyme resistant to feedback inhibition. The synthesis of this isoenzyme is still repressible by tryptophan; a consequence of this is that strain JP66 grows at a slower rate in glucose minimal medium supplemented with tryptophan than in unsupplemented glucose minimal medium, and we assume under these conditions growth rate is limited by the activity of the repressed level of DAHP synthetase Trp. There is excellent agreement between the repressed level of DAHP synthetase found in extracts of JP66 and the in vivo rate of phenylalanine plus tyrosine biosynthesis which we calculate from their growth rate data to occur in cells of JP66 grown in the presence of tryptophan.)

The relative rates of enzyme synthesis per gene found when wild-type cells were grown in medium 17AA indicate that under these conditions completely derepressed synthesis of DAHP synthetase Tyr occurred (Table 3), which contrasts with the low relative rate of synthesis of this isoenzyme in glucose minimal medium and suggests the intracellular concentration of free tyrosine (which is the end product involved in repression of DAHP synthetase Tyr) is lower in cells grown in medium 17AA than in glucose minimal medium.

Comparison of the specific activity of DAHP synthetase Tyr found in extracts of cells grown in medium 17AA with estimated rates of incorporation of aromatic amino acids into protein (Table 3, footnote *b*) suggests that (in spite of lowered intracellular tyrosine concentrations) the in vivo rate of DAHP synthesis is less than 2% of the rate found in cell extracts, again suggesting that a considerable degree of feedback inhibition occurs in these cells or that perhaps the in vivo reaction velocity is limited by the concentration of the substrates phosphoenolpyruvate or D-erythrose 4-phosphate.

The enzyme levels of DHQ dehydratase, EPSP synthetase, and chorismate synthetase are shown to be lower when cells were grown in medium 17AA than if the cells were grown in glucose minimal medium (Table 4). This argues against the activity of these enzymes having a controlling effect on the flow of intermediates along the pathway under these conditions and shows that these enzymes operate at less than maximum velocity in cells growing in glucose minimal medium. This inference is supported by the finding that, with the exception of shikimate kinase and chorismate synthetase, the values of enzyme activity found in extracts of cells grown in glucose minimal medium are many fold in excess of the estimated rate of aromatic biosynthesis occurring at these growth rates (see Tables 3 and 4 and the footnotes to these tables).

With many repressible enzymes, the rate of synthesis in minimal medium is much less than the fully derepressed rate of synthesis (26), and this generalization applies to the data on DAHP synthetases Phe and Tyr and on shikimate kinase given in Table 3. Although earlier reports have shown that in regulator-defective cells there is a greater capacity for derepression of DAHP synthetase Tyr than for the other DAHP synthetase isoenzymes (30), the data in Table 3 provide the first demonstration of a situation in which DAHP synthetase Phe is not the major activity in wild-type cells and provide an example of a situation in which the capacity of a wild-type cell for derepression of a biosynthetic enzyme is fully utilized in response to a requirement for increased rate of end product synthesis.

ACKNOWLEDGMENTS

This work was supported by a grant from the Australian Research Grants Committee. D. E. Tribe and Helen Camakaris hold Commonwealth postgraduate research awards.

We are most grateful to Sally Morrison, B. Davidson, and L. Sparrow for help in purification of chemicals and to J. Camakaris and B. Ely for permission to use published observations.

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