

REGULATION OF AROMATIC AMINO ACID BIOSYNTHESIS IN *ESCHERICHIA COLI* K12¹

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THE pathway of aromatic amino acid biosynthesis in *Escherichia coli* is shown in Figure 1.

The first reaction of this pathway, the formation of 3-deoxy-D-arabinoheptulosonic acid 7-phosphate (DAHP) is catalyzed in *E. coli* by three separable DAHP synthetases (DOY and BROWN 1965; WALLACE and PITTARD 1967a, b). One DAHP synthetase is feedback-inhibited almost completely by phenylalanine and partially by tyrosine; another is inhibited almost completely by tyrosine. The third DAHP synthetase is not inhibited by any one of the aromatic amino acids but there is some evidence that it may be partially inhibited by tryptophan (DOY 1967; BROWN unpublished results).

Earlier experiments in *E. coli* W (BROWN and DOY 1966) had shown that the phenylalanine-inhibited DAHP synthetase is repressed by exogenous phenylalanine or tryptophan while the tyrosine-inhibited DAHP synthetase is repressed by low concentrations of exogenous tyrosine and by high concentrations of phenylalanine or tryptophan. In these experiments the possibility of multivalent repression of DAHP synthetase could not be directly tested. The present paper reports similar repression properties for wild-type *E. coli* K12. In addition, experimentation with aromatic auxotrophs of *E. coli* K12 in the chemostat reveals multivalent repression of one of the DAHP synthetases. Repression of several other enzymes of the aromatic pathway is also reported as a preliminary to obtaining an integrated picture of repression for the whole aromatic pathway.

The occurrence of three discrete DAHP synthetases, each regulated in a specific fashion by feedback inhibition and repression, raised the possibility of separate structural and regulatory genes for these enzymes and prompted the genetic and biochemical studies reported here.

Two classes of mutants lacking individual DAHP synthetases were obtained by applying the principle that, if the activity of two of the three DAHP synthetases could be minimized through feedback inhibition and repression by two of the aromatic amino acids, then a mutation affecting the third DAHP synthetase would result in a requirement for the third aromatic amino acid. Such mutants require one of the aromatic amino acids when the other two aromatic amino acids are present but do not require it when the other two amino acids

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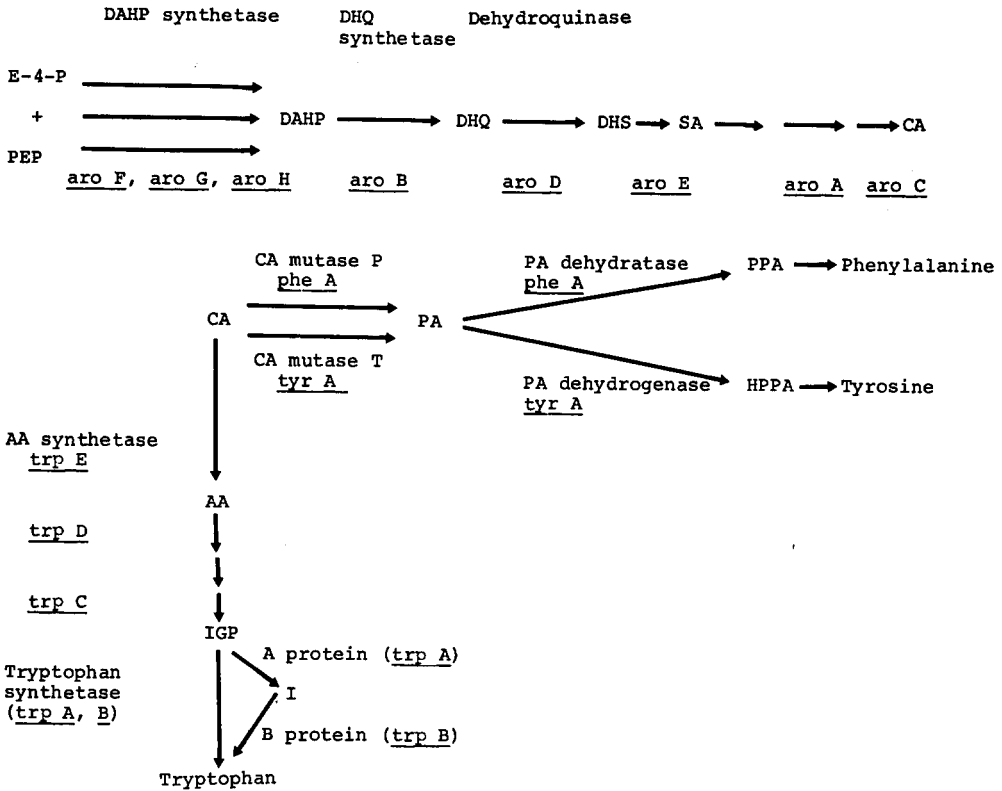


FIGURE 1.—The aromatic amino acid pathway in *E. coli* K12. The genetic symbols for each of the known structural genes are included. E-4-P, erythrose-4-phosphate; PEP, phosphoenolpyruvate; DAHP, 3-deoxy-D-arabino-heptulosonic acid 7-phosphate; DHQ, dehydroquininate; DHS, dehydroshikimate; SA, shikimate; CA, chorismate; AA, anthranilate; IGP, indole-3-glycerol phosphate; I, indole; PA, prephenate; PPA, phenylpyruvate; HPPA, hydroxyphenylpyruvate.

are absent. One of the classes isolated lacked the phenylalanine-inhibited DAHP synthetase, the other lacked the tyrosine-inhibited DAHP synthetase. A brief note reporting the isolation of these mutants has previously appeared (BROWN and MAAS 1966). The symbols *aro F* and *aro G* have been adopted to denote genes specifying the tyrosine-inhibited and phenylalanine-inhibited DAHP synthetases, respectively. Otherwise, the genetic symbols used by TAYLOR and THOMAN (1964) as modified by PITTARD and WALLACE (1966) and DEMEREC *et al.* (1966) have been employed.

Since one of the DAHP synthetases is repressed by tryptophan, it was of interest to determine whether a mutation in the tryptophan regulator gene (denoted *trp R* here, but formerly denoted *R try*) which prevents repression of the tryptophan operon by tryptophan (COHEN and JACOB 1959; ITO and CRAWFORD 1965) has a similar effect on the tryptophan-repressed DAHP synthetase. Examination of *trp R*⁻ strains obtained from DR. F. JACOB and DR. I. P. CRAWFORD

showed that, in these strains, the tryptophan-repressed DAHP synthetase is, in fact, not repressible.

MATERIALS AND METHODS

Organisms: All strains used in this work are derivatives of *E. coli* K12 and are described in Table 1. The origins and directions of transfer of the Hfr strains used in mating experiments are shown in Figure 4. Wild type, W1485, was provided by DR. R. L. SOMERVILLE. DR. A. L. TAYLOR provided strains, AT1360, AT2022 and AT2471. The *aro D*⁻ allele in AT1360 confers a requirement for phenylalanine, tyrosine, tryptophan, *p*-aminobenzoic acid and *p*-hydrobenzoic acid. Strain P118 was provided by DR. P. REEVES. DR. B. LOW provided strains KL19, KL16, Hfr-HAYES and HfrCAVALLI. DR. E. MCFALL provided strain Hfr 6 and transducing phage, P1*k*c. DR. F. JACOB provided strain GC19 and DR. I. P. CRAWFORD provided strains 829 and 687. 687 has not been fully characterized; it carries the *trp R*⁻ allele and requires a growth factor present in casein hydrolysate. DR. W. HAUT isolated strain KB5000. This strain is an *aro*⁻ derivative of strain JC1553. The latter has been described by CLARK and MARGULIES (1965). The aromatic block in KB5000 is distal to 5-dehydroquinase and causes a quintuple aromatic requirement similar to that of AT1360 (BROWN unpublished results). Strains KB1100 and KB1200 were isolated in this laboratory.

Media: The basal medium was medium A of DAVIS and MINGIOLI (1950). The synthetic enriched (SE) medium was the medium AF of NOVICK and MAAS (1961) modified by the inclusion of arginine (100 µg/ml) and the substitution of glucose (0.2%) for lactate.

Growth: Cultures were grown at 37°C with shaking on New Brunswick gyrotory shakers. Growth was estimated by sampling and reading the absorbancy at 490 mµ in a 1 cm cuvette with a Beckman DU spectrophotometer.

Preparation of cell-free extracts and estimation of protein: The preparation of cell-free

TABLE 1

List of strains

Strain	Genetic loci	Sex
W1485	wild type, <i>str-s</i>	F+
AT1360	<i>aro D</i> ⁻ , <i>pro</i> ⁻ , <i>lac</i> ⁻ , <i>tsx</i> ⁻ , <i>gal</i> ⁻ , <i>his</i> ⁻ , <i>xyl</i> ⁻ , <i>mtl</i> ⁻ , <i>arg</i> ⁻ , <i>thi</i> ⁻ , (λ) ⁻	F-
KB5000	<i>aro</i> ⁻ , <i>leu</i> ⁻ , <i>his</i> ⁻ , <i>arg</i> ⁻ , <i>met</i> ⁻ , <i>lac</i> ⁻ , <i>mal</i> ⁻ , <i>str-r</i> , <i>rec</i> ⁻	F-
KB1100	<i>aro G</i> ⁻ , <i>str-r</i>	F-
KB1200	<i>aro F</i> ⁻ , <i>str-r</i>	F-
AT2022	<i>phe A</i> ⁻ , <i>pro</i> ⁻ , <i>his</i> ⁻ , <i>arg</i> ⁻ , <i>lac</i> ⁻ , <i>tsx</i> ⁻ , <i>gal</i> ⁻ , <i>xyl</i> ⁻ , <i>mtl</i> ⁻ , <i>mu</i> ⁺ , <i>thi</i> ⁻	F-
AT2471	<i>tyr A</i> ⁻ , <i>thi</i> ⁻ , (λ) ⁻	Hfr
P118	<i>his</i> ⁻ , <i>thr</i> ⁻ , <i>leu</i> ⁻ , <i>pro</i> ⁻ , <i>arg</i> ⁻ , <i>cim</i> ⁻ , <i>gal</i> ⁻ , <i>ara</i> ⁻ , <i>lac</i> ⁻ , <i>str-r</i> , <i>thi</i> ⁻ , (λ) ⁻	F-
KL19	<i>str-s</i>	Hfr
KL16	<i>thi</i> ⁻ , <i>str-s</i>	Hfr
Hfr 6	<i>met</i> ⁻ , <i>str-s</i>	Hfr
HfrHAYES	<i>thi</i> ⁻ , <i>str-s</i>	Hfr
HfrCAVALLI	<i>str-s</i>	Hfr
GC19	<i>trp R</i> ⁻ , <i>thi</i> ⁻	Hfr
829	<i>trp R</i> ⁻ , <i>cys B</i> ⁻	...

The following abbreviations are used: *aro*, aromatic amino acids and vitamins; *tsx*⁻, resistance to bacteriophage T6; *str*, streptomycin; *s*, sensitive; *r*, resistant; *pro*, proline; *thi*, thiamine; *arg*, arginine; *his*, histidine; *thr*, threonine; *leu*, leucine; *phe*, phenylalanine; *tyr*, tyrosine; *trp*, tryptophan; *mtl*, mannitol; *xyl*, xylose; *met*, methionine; *lac*, lactose; *gal*, galactose; *ara*, arabinose; (λ), lysogenic for bacteriophage λ; *mu*, mutator bacteriophage; *cim*, colicin immunity; *trp R*, tryptophan regulatory gene; *cys*, cysteine. For a description of the enzymes affected by mutations in the *aro D*, *aro F*, *aro G*, *phe A* and *tyr A* genes see Figure 1.

extracts has been previously described (Doy and Brown 1965). Protein was estimated by the method of Lowry *et al.* (1951).

Chemicals: Inorganic chemicals were of A. R. grade. The aromatic amino acids used were the L-isomers. Organic chemicals were of the highest grade commercially available. D-erythrose-4-phosphate dimethylacetal dicyclohexylammonium salt was purchased from the California Corporation for Biochemical Research and converted to D-erythrose-4-phosphate by the method of BALLOU, FISHER and MACDONALD (1955). Chorismic acid was prepared as the barium salt from the supernatant fluid of *Aerobacter aerogenes* 62-1 by the method of F. GIBSON (1964) who also provided this strain. A sample of DAHP (barium salt) was the gift of D. B. SPRINSON. Crude colicin E was prepared from the colicinogenic strain CA42 by the method of REEVES (1963) and stored at -20°C .

Enzyme assays: DAHP synthetase: This enzyme was measured by the method of Doy and BROWN (1965). The turbidity previously encountered in the assay of DAHP was largely avoided by maintaining the DAHP-assay reaction mixture at 37°C after heating in the boiling water bath until the absorbancy at $549\text{ m}\mu$ was measured. One unit of DAHP synthetase is defined as the amount of enzyme that catalyzes the formation of $1\text{ m}\mu\text{mole}$ of DAHP per min at 37°C . The concentration of DAHP was calculated using the value of 5.3×10^4 for the molar extinction coefficient at $549\text{ m}\mu$ (measured on a sample taken from the complete DAHP synthetase reaction mixture (protein included)). This value is based on a mol. wt. of 527 for DAHP (empirical formula: $\text{C}_7\text{H}_{10}\text{O}_{10}\cdot\text{P}\cdot 1.5\text{Ba}\cdot 2\text{H}_2\text{O}$) and assumes 100% purity for the reference standard. The method of estimating the activities of individual DAHP synthetases in crude extracts has been previously described (BROWN and Doy 1966). The validity of these estimates has been established in crude extracts of *E. coli* W by $(\text{NH}_4)_2\text{SO}_4$ fractionation and assay of the separate DAHP synthetases (Doy and BROWN 1965) and confirmed in *E. coli* K12 by similar fractionations (BROWN unpublished results).

5-Dehydroquinase synthetase: The method of SRINIVASAN, ROTHSCCHILD and SPRINSON (1963) was used. One unit of 5-dehydroquinase synthetase is defined as that amount of enzyme which causes the disappearance of $0.1\text{ }\mu\text{mole}$ of DAHP in 30 min at 37°C .

5-Dehydroquinase: The method of MITSUHASHI and DAVIS (1954) was used. The substrate for the reaction was the supernatant fluid containing the accumulation products (chiefly 5-dehydroquinic acid) of *E. coli* 83-1 (obtained from Dr. B. D. DAVIS). One unit of 5-dehydroquinase is defined as that amount of enzyme that catalyzes the formation of $1.0\text{ }\mu\text{mole}$ of 5-dehydroshikimic acid in 1 min under reaction conditions.

Chorismate mutase: The method of COTTON and GIBSON (1965) was used. The specific activity of chorismate mutase is the increase in absorbancy at $320\text{ m}\mu$ in 20 min per mg of protein at 37°C . For the assay of crude cell extracts 0.1 ml of an appropriate dilution of the extract was used.

Tryptophan synthetase: The method used was essentially that of SMITH and YANOFSKY (1962). One unit of enzyme is that amount which causes the disappearance of $0.1\text{ }\mu\text{mole}$ of indole in 20 min at 37°C .

$(\text{NH}_4)_2\text{SO}_4$ fractionation of crude extracts: The procedure of Doy and BROWN (1965) was followed.

Repression of enzymes of aromatic biosynthesis: repression in growing, wild-type E. coli K12: The basal growth medium was supplemented with aromatic amino acids as indicated in RESULTS. Cells were harvested in exponential phase (absorbancy at $490\text{ m}\mu$, 0.6) extracts were prepared and the specific activities of the enzymes measured.

Derepression in a multiple aromatic auxotroph grown in the chemostat with limitation by single aromatic amino acids: The multiple aromatic auxotroph AT1360, which is blocked in 5-dehydroquinase, was used in all chemostat experiments with the exception of the repression studies of 5-dehydroquinase, in which case KB5000 was used. Cells were maintained in the chemostat at a density of 5×10^8 cells/ml (absorbancy at $490\text{ m}\mu$, 0.6) by supplying at a constant, growth-limiting rate, a single aromatic amino acid, contained in basal medium supplemented with an excess of the two remaining aromatic amino acids and other growth requirements. Volume of the culture was 500 ml and the rate of supply of limiting nutrient was 100 ml/hour .

The generation time was 5 hours. The rates of inflow of fresh medium and outflow of culture were controlled with a Sigma pump (model T-8). Aeration and mixing of the culture were achieved by shaking on a New Brunswick gyrotory shaker. The cells pumped out were collected in a sterile flask held in an ice-bath and harvested at intervals. Crude extracts were prepared and the specific activities of enzymes measured. Values reported are steady-state values. In practice, these values were reached after three generations in the chemostat.

Isolation of mutants lacking individual DAHP synthetases: The technique described is based on the methods of ADELBERG, MANDEL and CHEN (1965) and SOMERVILLE (personal communication). An F⁻, streptomycin-resistant derivative of W1485 was obtained by curing with acridine orange (after the method of HIROTA 1960) and direct selection on agar medium containing streptomycin (100 µg/ml). An overnight culture of this derivative was sub-cultured into 10 ml of SE medium. This culture was grown with shaking to exponential phase (5×10^8 cells/ml) then centrifuged and the pellet washed with 10 ml of 0.025M Tris-maleate buffer (pH 6.0). After centrifugation, the washed pellet was resuspended in 10 ml of 0.025M Tris-maleate buffer (pH 6.0) in a 125 ml Erlenmeyer flask and treated with N-methyl-N'-nitro-N-nitrosoguanidine (final concentration, 100 µg/ml). This suspension was incubated at 37° with shaking in a New Brunswick gyrotory, water-bath shaker, for 30 min. After incubation, 5 ml of the suspension was centrifuged, washed once with cold basal medium and resuspended in 5 ml of basal medium. A 1.0 ml sample was then inoculated into 5 ml of SE medium and incubated with shaking at 37°C for 5 hrs. The culture was then centrifuged, washed with 5 ml of SE medium *minus* the aromatic amino acids and resuspended in 5 ml of the same medium. These cells were then exposed to penicillin (300 units/ml) at a cell density of 10^7 /ml in 5 ml volumes of SE medium *minus* the aromatic amino acids, each supplemented with a different pair of the aromatic amino acids at a concentration of 2×10^{-4} M each. These suspensions were incubated with shaking at 37°C for 120 min. Penicillin was then removed by centrifugation and appropriate dilutions of the cells spread on agar plates containing SE medium *minus* the aromatic amino acids and incubated 48 hrs at 37°C. Colonies were then replicated onto agar plates containing the same medium supplemented with pairs of the aromatic amino acids (concentration, 2×10^{-4} M each). Colonies which failed to grow on these plates were then sub-cultured and purified.

Mating conditions and interruption of mating: The method described by Low and Wood (1965) was used. The blending liquid was cold, 0.9% saline.

P1kc transduction techniques and preparation of phage stocks: The techniques used were essentially those of LENNOX (1955). The medium used was the L-broth of LURIA and BURROUS (1957). CaCl₂ was added to this medium at a final concentration of 5×10^{-3} M to facilitate phage adsorption.

RESULTS

Repression of DAHP synthetase: It can be seen from Tables 2 and 3 that the phenylalanine-inhibited DAHP synthetase was derepressed when growth of AT1360 was limited by either phenylalanine or tryptophan, indicating that both of these amino acids are required for repression of this enzyme.

The tyrosine-inhibited DAHP synthetase was derepressed when growth of AT1360 was limited by tyrosine and the concentration of phenylalanine and tryptophan was 10^{-4} M (Table 2). When the concentration of phenylalanine and tryptophan was 10^{-3} M, the extent of derepression of the tyrosine-inhibited DAHP synthetase was greatly reduced (Table 3). The highest level of derepression of this enzyme occurred when growth of AT1360 was limited by shikimic acid, an intermediate of the common aromatic pathway (Table 2).

The third DAHP synthetase was derepressed when growth of AT1360 was limited by tryptophan (Tables 2 and 3).

Table 2 also summarizes the results of experiments with wild-type *E. coli* K12

TABLE 2

Repression of enzymes of aromatic biosynthesis in wild-type E. coli K12 (W1485) and the multiple aromatic auxotroph AT1360

Strain	Supplements (all $10^{-4}M$)	Limiting growth factor	Enzyme activity (units/mg protein)					
			DAHP synthetases			DHQ synthetase	Chorismate mutase	Trp synthetase
			Phe- inhibited	Tyr- inhibited	Trp- repressed			
AT1360	Phe+Tyr+Trp	...	25.4	0.0	1.2	4.0	1.6	0.3
AT1360	Tyr+Trp	Phe	100.3	0.0	1.7	7.1	17.5	0.16
AT1360	Phe+Trp	Tyr	24.4	7.0	1.6	6.4	2.3	0.3
AT1360	Phe+Tyr	Trp	120.0	0.0	3.2	6.0	1.6	22.6
AT1360	None	SA	71.0	83.0	2.0	6.0	15.4	..
W1485	None	...	41.0	0.8	1.7	..	2.9	0.4
W1485	Phe	...	16.2	2.0	2.0
W1485	Trp	...	28.6	0.6	0.8	0.2
W1485	Phe+Tyr	...	38.4	0.0	3.2	2.9
W1485	Phe+Tyr+Trp	...	24.3	0.0	0.7	..	1.7	0.3

Methods for the growth of bacteria, and the measurement of enzymes are described in MATERIALS and METHODS. When AT1360 was grown in the chemostat, the concentration of limiting Phe and Tyr was $2 \times 10^{-5}M$; limiting Trp was $5 \times 10^{-6}M$ and limiting SA, 10 $\mu g/ml$. The culture of AT1360 supplemented with Phe + Tyr + Trp was harvested in exponential phase, at a cell density of $5 \times 10^8/ml$. The method for studying repression in wild-type *E. coli* K12 (W1485) is described in MATERIALS and METHODS.

Abbreviations: DHQ, 5-dehydroquinate; Phe, phenylalanine; Tyr, tyrosine; Trp, tryptophan; SA, shikimate.

strain W1485. In this organism the phenylalanine-inhibited DAHP synthetase was the major component of total DAHP synthetase activity under all growth conditions investigated. Exogenous phenylalanine ($10^{-4}M$) repressed the phenylalanine-inhibited DAHP synthetase and caused slight derepression of the tyrosine-inhibited enzyme. Phenylalanine *plus* tyrosine repressed the tyrosine-inhibited DAHP synthetase and derepressed the phenylalanine-inhibited and tryptophan-repressed DAHP synthetases relative to the levels in cells grown in the presence of phenylalanine alone. Phenylalanine *plus* tyrosine *plus* tryptophan repressed all three DAHP synthetases.

TABLE 3

Repression of DAHP synthetase in AT1360

Supplements (all $10^{-3}M$)	Limiting growth factor	Specific activity of DAHP synthetases		
		Phe- inhibited	Tyr- inhibited	Trp- repressed
Phe+Tyr+Trp	...	29.4	0.0	0.8
Tyr+Trp	Phe	112.4	0.0	1.6
Phe+Trp	Tyr	18.8	1.6	0.8
Phe+Tyr	Trp	122.4	0.0	2.6

Except for the concentration of non-limiting aromatic amino acids, which in these experiments was $10^{-3}M$, the experimental details were similar to those for AT1360 in Table 2.

Repression of 5-dehydroquinase synthetase: This enzyme, which catalyzes the second reaction of the aromatic pathway was derepressed only slightly in AT1360 when growth was limited by each of the aromatic amino acids in turn (Table 2). The extent of derepression varied from approximately 1.8-fold in the case of phenylalanine limitation to approximately 1.5-fold for tyrosine or tryptophan limitation.

Repression of 5-dehydroquinase: Little repression of this enzyme (the third of the aromatic pathway) was detected in KB5000 (Table 4). The specific activities of extracts of cells grown under conditions of aromatic amino acid limitation were only about 25% higher than the repressed control.

Repression of chorismate mutase: Growth limitation by phenylalanine derepressed overall chorismate mutase activity (Table 2). Tyrosine limitation also caused slight derepression but tryptophan limitation had no effect.

The concentration of chorismate mutase is low in extracts of W1485 grown on basal medium. This concentration is only 70% higher than that in W1485 grown in the presence of phenylalanine *plus* tyrosine *plus* tryptophan (Table 2). Thus, it appears that chorismate mutase is relatively repressed in wild-type cells grown in basal medium. This presumably, is because the intracellular concentrations of endogenously synthesized phenylalanine and/or tyrosine exceed those required for repression.

Repression of tryptophan synthetase: Tryptophan synthetase was derepressed approximately 75-fold when growth of AT1360 was limited by tryptophan (Table 2). This derepression was not coordinate with that of the tryptophan-repressed DAHP synthetase which was derepressed about 3-fold by tryptophan limitation. Tryptophan synthetase also was derepressed in W1485 when this organism was grown in the presence of phenylalanine *plus* tyrosine. The addition of tryptophan to this medium repressed the enzyme (Table 2). Derepression of tryptophan pathway enzymes by growth in the presence of phenylalanine *plus* tyrosine was previously observed by SOMERVILLE (personal communication).

Biochemical analysis of mutant strains blocked in individual DAHP synthetases: The mutation isolation technique described in MATERIALS AND METHODS yielded two classes of mutants typified by strains KB1100 and KB1200. KB1100 grew on SE medium *minus* the aromatic amino acids and on basal medium.

TABLE 4

Specific activities of 5-dehydroquinase in KB5000

Supplements	Limiting aromatic growth factor	5-dehydroquinase activity (units/mg protein)
Phe+Tyr+Trp	None	0.048
Tyr+Trp	Phe	0.053
Phe+Trp	Tyr	0.061
Phe+Tyr	Trp	0.057

KB5000 is described in MATERIALS AND METHODS. The same experimental procedure was followed as described in Table 2 for AT1360. 5-Dehydroquinase was measured as described in MATERIALS AND METHODS.

Growth was inhibited if tyrosine and tryptophan were included in these media. Growth of the wild-type parent, W1485, was not similarly affected. Either phenylalanine or shikimic acid relieved the inhibition of KB1100 by tyrosine *plus* tryptophan. KB1200 grew on SE medium *minus* the aromatic amino acids and on basal medium but was inhibited in the presence of phenylalanine *plus* tryptophan. Growth of W1485 was not similarly affected. Either tyrosine or shikimic acid relieved the inhibition of KB1200 by phenylalanine *plus* tryptophan. These nutritional responses suggested that in the presence of tyrosine *plus* tryptophan, and phenylalanine *plus* tryptophan, KB1100 and KB1200, respectively, were blocked in the aromatic pathway before shikimic acid.

Examination of the patterns of feedback inhibition of DAHP synthetase in crude extracts revealed the absence of phenylalanine-inhibited DAHP synthetase activity in KB1100 and the absence of tyrosine-inhibited DAHP synthetase activity in KB1200 (Table 5). This was confirmed by $(\text{NH}_4)_2\text{SO}_4$ fractionation of these crude extracts. (Figure 2). No phenylalanine-inhibited DAHP synthetase activity could be detected in $(\text{NH}_4)_2\text{SO}_4$ fractions of KB1100 (Figure 2b). Similarly, $(\text{NH}_4)_2\text{SO}_4$ fractions of KB1200 lacked the tyrosine-inhibited DAHP synthetase (Figure 2c). Figure 2a shows the fractionation profile of the wild-type parent, W1485.

Genetic analysis of mutant strains blocked in individual DAHP synthetases: The approximate position on the chromosome of the gene specifying the phenylalanine-inhibited DAHP synthetase (*aro G*) was found by mating the *aro G*⁻ recipient, KB1100, with a number of different Hfr donors. Mating was interrupted in all cases at 30 minutes. By this time, the wild-type *aro G*⁺ allele had been transferred to KB1100 by HfrHAYES and Hfr 6 but not by HfrCAVALLI or KL19. The known origins and directions of transfer of these Hfr strains are shown in Figure 4. From these data it was deduced that the *aro G* gene lies in the segment of the chromosome (approximately 6 minutes long on the time scale)

TABLE 5

The specific activities of enzymes of aromatic biosynthesis in wild-type E. coli K12 and derived mutants

Strain	Supplements	Enzymes			
		DAHP synthetases			Tryptophan synthetase
		Phe-inhibited	Tyr-inhibited	Trp-repressed	
KB1100	None	0.0	3.2	2.3	..
KB1200	Phe	19.8	0.0	2.6	..
GC19	None	38.4	0.7	3.5	6.7
GC19	Trp	27.3	0.5	4.2	5.9
GC19	Phe+Tyr+Trp	26.0	0.0	4.5	6.8

For details of growth, extract preparation and enzyme estimations see MATERIALS AND METHODS. The strains are described in Table 1 and in the text. GC19 has a requirement for thiamine; this was provided at a concentration of 1 $\mu\text{g}/\text{ml}$. Abbreviations: Phe, phenylalanine; Tyr, tyrosine; Trp, tryptophan.

TABLE 6

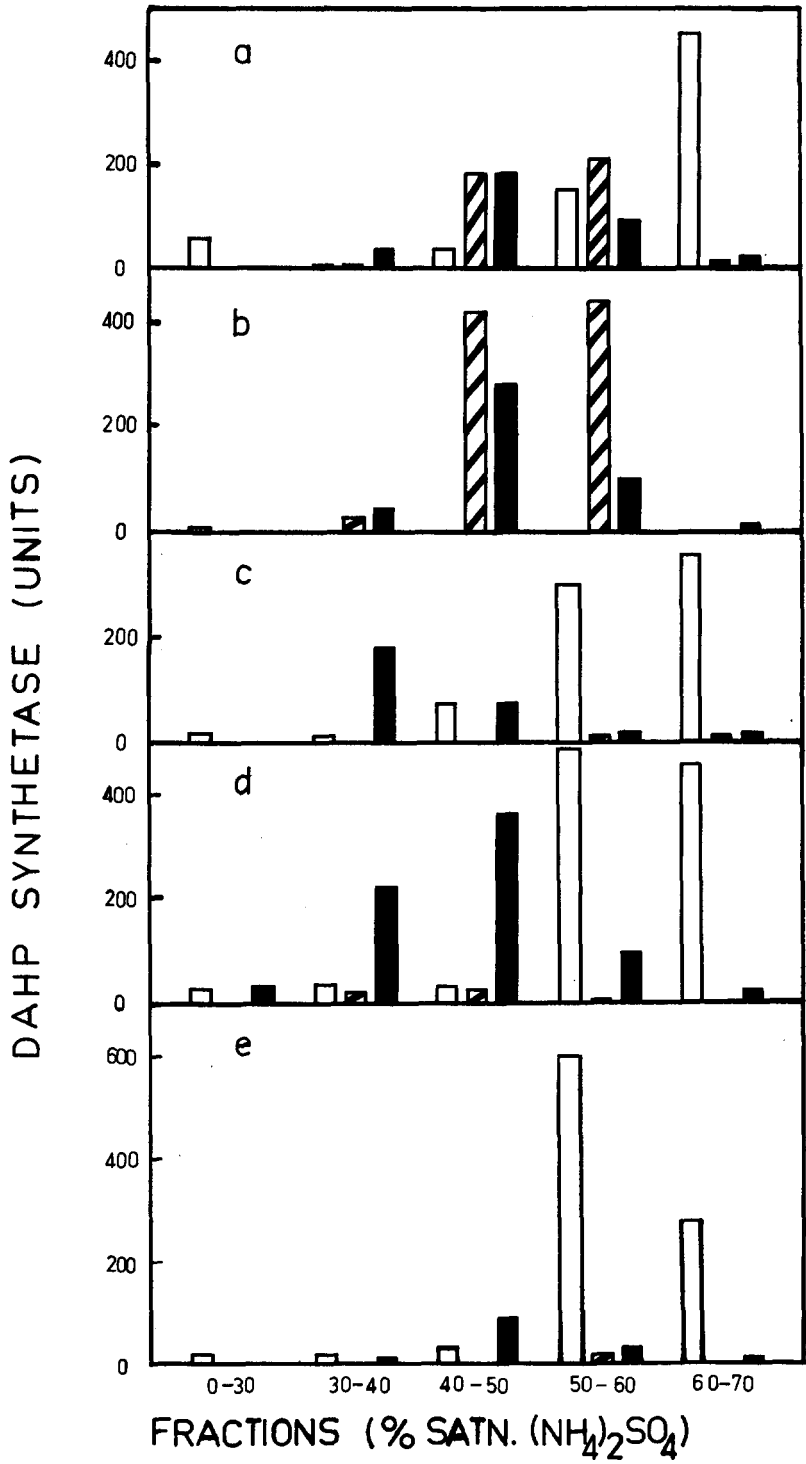
Cotransduction of *aro G* with *gal* and *cim*, and cotransduction of *aro F* with *tyr A* and *phe A*

Transductants	Cross		
	P1 <i>kc</i> P118 × KB1100	P1 <i>kc</i> KB1200 × AT2471	P1 <i>kc</i> KB1200 × AT2022
<i>aro G</i> ⁺ selected (Number)	125
<i>aro G</i> ⁺ <i>gal</i> ⁻ unselected (Number)	113
<i>aro G</i> ⁺ <i>cim</i> ⁻ unselected (Number)	106
<i>tyr A</i> ⁺ selected (Number)	...	126	...
<i>tyr A</i> ⁺ <i>aro F</i> ⁻ unselected (Number)	...	82	...
<i>phe A</i> ⁺ selected (Number)	267
<i>phe A</i> ⁺ <i>aro F</i> ⁻ unselected (Number)	133

Transduction techniques and preparation of phage lysates are described in MATERIALS AND METHODS. The strains used in these crosses are described in Table 1. Multiplicity of infection for transduction was 5.0; recipient cell density was 2×10^8 /ml. *aro G*⁺ recombinants were selected on basal medium containing tyrosine *plus* tryptophan (2×10^{-4} M each), then replicated onto basal medium containing galactose (2%) instead of glucose to test for unselected *gal*⁻ transductants and onto basal medium containing colicin E (10 units/ml) to test for unselected *cim*⁻ transductants. The *cim*⁻ allele confers resistance to colicins E and K (P. REEVES personal communication). *tyr A*⁺ and *phe A*⁺ recombinants were selected on media containing an excess of all growth requirements for the recipient except for tyrosine and phenylalanine, respectively. Recombinants were then replicated on to media containing phenylalanine *plus* tryptophan (2×10^{-4} M) to test for unselected *aro F*⁻ recombinants.

between the origins of HfrCAVALLI and KL19. The close linkage of *aro G* with the galactose region and the *cim* locus was established by the P1*kc*-mediated transductions reported in Table 6. The *aro G*⁺ allele of P118 was cotransduced with the *gal*⁻ and *cim*⁻ alleles of this donor with frequencies of 90% and 85%, respectively. The *cim*⁻ allele confers resistance to colicin E and is 75% cotransducible with the galactose region (P. REEVES personal communication). This value has been confirmed in this laboratory. These data indicate that *aro G* is located between the *cim* locus and the galactose region. It is not yet known on which side of *gal* the *cim* and *aro G* loci lie, hence the locations shown in Figure 4 are arbitrary.

The approximate position of the gene specifying the tyrosine-inhibited DAHP synthetase (*aro F*) was found by mating the *aro F*⁻ recipient, KB1200, with several Hfr strains. The Hfr strain which transferred *aro F*⁺ with the highest frequency (KL16) was chosen as the donor for an interrupted mating experiment with KB1200. The origin and direction of transfer of KL16 are shown in Figure 4. KL16 injected *aro F*⁺ 12 minutes after the commencement of mating (Figure 3). This suggested that *aro F* lies near the *phe A* and *tyr A* genes. The results of cotransduction tests of *aro F* with the *phe A* and *tyr A* genes are shown in Table 6. The *aro F*⁻ allele of KB1200 is 65% cotransducible with the *tyr A*⁺ allele and 50% cotransducible with the *phe A*⁺ allele. These results are essentially in agreement with the cotransduction frequencies reported previously by PITTARD and WALLACE (1966) for a mutation conferring similar nutritional properties to that of an *aro F*⁻ allele. WALLACE and PITTARD (1967a) subsequently demonstrated that this mutation does, in fact, cause a loss of tyrosine-inhibited DAHP



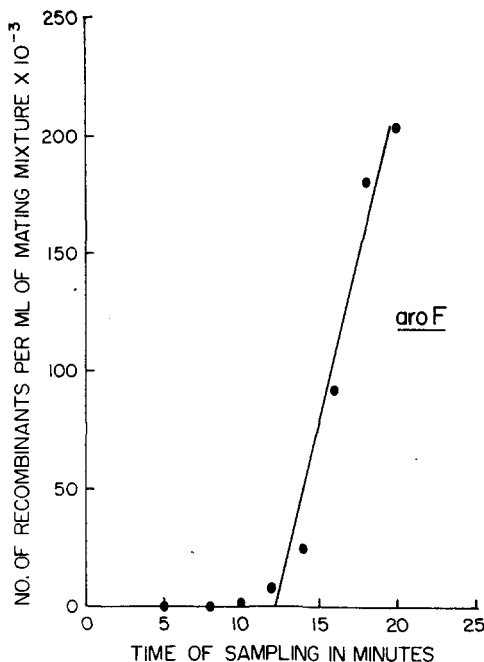


FIGURE 3.—Kinetics of zygote formation when the Hfr, KL16, is mated with the female, KB1200. Details of mating conditions and interpretation of mating are described in MATERIALS AND METHODS. Samples of the interrupted mating mixture were spread on basal medium containing phenylalanine *plus* tryptophan ($2 \times 10^{-4}M$ each) and streptomycin (100 $\mu g/ml$).

synthetase activity. The positions of *aro G* and *aro F* relative to other genes of aromatic biosynthesis are shown on the map of the *E. coli* chromosome in Figure 4. The relative positions of *tyr A* and *phe A* shown here are arbitrary.

Biochemical analysis of mutants sensitive to phenylalanine plus tyrosine: Mutants isolated on the basis of sensitivity to the pair phenylalanine *plus* tyrosine were not obtained in this laboratory; however, two isolated by DR. R. L. SOMERVILLE, were examined. These mutants could grow on basal medium but were

FIGURE 2 (a-e).—Distribution of DAHP synthetase activity in $(NH_4)_2SO_4$ fractions of extracts of wild-type *E. coli* K12 (W1485) and strains KB1100, KB1200, GC19 and AT1360. For experimental details see MATERIALS AND METHODS. Distributions are based on analysis of 160 mg protein measured in the protamine sulphate supernatant. All crude extracts fractionated were obtained from cells grown on the media indicated and harvested in exponential phase (absorbancy at 490 $m\mu$, 0.6). FIGURE 2a.—Extract of W1485 grown on basal medium supplemented with phenylalanine ($10^{-4}M$). FIGURE 2b.—Extract of KB1100 grown on basal medium. FIGURE 2c.—Extract of KB1200 grown on basal medium supplemented with phenylalanine ($10^{-4}M$). FIGURE 2d.—Extract of GC19 grown on basal medium supplemented with thiamine (1 $\mu g/ml$) and phenylalanine, tyrosine and tryptophan ($10^{-4}M$ each). FIGURE 2e.—Extract of AT1360 grown on basal medium supplemented with phenylalanine, tyrosine, and tryptophan ($10^{-4}M$ each) together with an excess of all the other growth requirements of this strain (see Table 1). Open area, phenylalanine-inhibited DAHP synthetase; hatched area, tyrosine-inhibited DAHP synthetase; solid area, tryptophan-repressed DAHP synthetase.

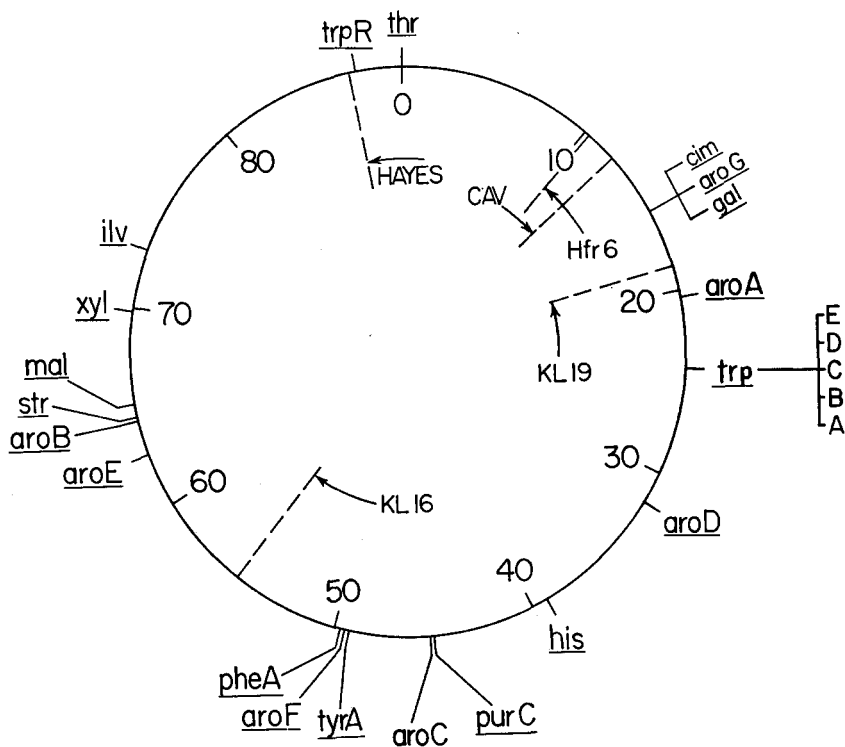


FIGURE 4.—Map of *E. coli* chromosome showing locations of aromatic genes and origins and directions of transfer of Hfr strains used in this work. The functions of the various aromatic genes are indicated in Figure 1 and are discussed in the text. The map is graduated in 10-minute intervals (89 minutes total) and is constructed from the author's data and those of TAYLOR and THOMAN (1964) and PITTARD and WALLACE (1966). The origins and directions of transfer of the Hfr strains KL16 and KL19 were determined by Low (1965, and personal communication, respectively). HfrHAYES and HfrCAVALLI were described by HAYES (1953) and CAVALLI-SFORZA (1950), respectively. Hfr 6 transfers *tsx* as an early marker in a counter-clockwise direction and has a similar origin and direction of transfer to strains B8 and B11 of BRODA (see HAYES 1964) and L13 of SNEATH (1962).

inhibited in the presence of phenylalanine *plus* tyrosine. Addition of tryptophan stimulated growth while shikimic acid relieved the inhibition without stimulating growth. This suggested that the inhibition occurs before shikimic acid in the aromatic pathway, however, these mutants were found to be normal with respect to the tryptophan-repressed DAHP synthetase (BROWN unpublished). SOMERVILLE (personal communication) observed that both these mutants have a partial block in the tryptophan pathway. This was confirmed by biochemical and genetic analysis in this laboratory. Several independently isolated, "leaky", tryptophan auxotrophs were then tested and found to be sensitive to phenylalanine *plus* tyrosine. It was also noted that the accumulation of intermediates of tryptophan biosynthesis by partially blocked tryptophan auxotrophs was strongly inhibited by phenylalanine *plus* tyrosine. So far, it appears to be a

general phenomenon that strains with a partial block in tryptophan biosynthesis are sensitive to phenylalanine *plus* tyrosine.

Biochemical analysis of trp R⁻ mutants: Some of the biochemical properties of GC19, a mutant shown by COHEN and JACOB (1959) to possess an altered tryptophan regulatory gene (*trp R*) are shown in Table 5. It can be seen that the synthesis of the tryptophan-repressed DAHP synthetase in GC19 is not repressible by tryptophan or by all three of the aromatic amino acids together. It was also confirmed that tryptophan synthetase cannot be repressed by tryptophan in GC19. Almost identical results to those shown for GC19 were obtained with crude extracts of two *trp R⁻* strains (829 and 687) obtained from DR. I. P. CRAWFORD. The high, constitutive level of the tryptophan-repressed DAHP synthetase in GC19 was confirmed by $(\text{NH}_4)_2\text{SO}_4$ fractionation of crude extracts and examination of the fractions for DAHP synthetase activity. An $(\text{NH}_4)_2\text{SO}_4$ fractionation pattern of an extract of GC19 grown in the presence of phenylalanine *plus* tyrosine *plus* tryptophan is shown in Figure 2d. The amount of DAHP synthetase activity in the 30–40% and 40–50% $(\text{NH}_4)_2\text{SO}_4$ fractions was approximately 7 times the amount in comparable fractions of a *trp R⁺* strain (AT1360) grown under similar conditions (Figure 2e).

DISCUSSION

Repression of the three DAHP synthetases is considerably more complicated than simply repression of each enzyme by a single, specific, aromatic amino acid. Firstly, repression of the phenylalanine-inhibited DAHP synthetase is multivalent, requiring both phenylalanine and tryptophan. This type of control is analogous to the multivalent repression of the threonine-inhibited aspartate kinase by threonine and isoleucine found in *Salmonella* and *E. coli* (FREUNDLICH 1963). Like the formation of DAHP, the synthesis of aspartyl phosphate is the first specific reaction of a branched pathway and, it too, is catalyzed by multiple enzymes (STADTMAN *et al.* 1961). Secondly, the tyrosine-inhibited DAHP synthetase, while most sensitive to repression by tyrosine may also be repressed by phenylalanine and tryptophan in high concentrations (10^{-3}M). The physiological significance of these latter effects is not yet clear. The only DAHP synthetase repressed by a single aromatic amino acid appears to be the tryptophan-repressed DAHP synthetase.

The failure of the tryptophan-repressed DAHP synthetase to be inhibited or repressed by phenylalanine and tyrosine ensures the continued biosynthesis of tryptophan and the aromatic vitamins in the presence of the former amino acids. Phenylalanine and tyrosine, however, do reduce the endogenous concentration of tryptophan below that required to repress tryptophan synthetase and the phenylalanine-inhibited and tryptophan-repressed DAHP synthetases. In the absence of alternative sites of control of tryptophan synthesis by phenylalanine and tyrosine, the most probable explanation for the decrease in tryptophan production is that phenylalanine and tyrosine block the formation of DAHP by the phenylalanine-inhibited and tyrosine-inhibited DAHP synthetases and that the

amount of DAHP formed by the third DAHP synthetase is insufficient to allow production of tryptophan at repressing levels. The reduction in the flow of aromatic intermediates towards tryptophan does not restrict the growth of wild-type *E. coli* but does restrict the growth of strains which have a partial block in the tryptophan pathway. The inhibition of these strains observed in the presence of phenylalanine *plus* tyrosine is, thus, the combined effect of reduced concentrations of aromatic intermediates and inability to utilize them efficiently for tryptophan biosynthesis.

The significance of the small increments in the levels of 5-dehydroquinase synthetase and 5-dehydroquinase observed upon aromatic amino acid limitation of multiple aromatic auxotrophs, AT1360 and KB5000, is uncertain. The inability to demonstrate repression of 5-dehydroquinase is in accord with an earlier finding in *E. coli* W that there is less than a 2-fold increase in the concentration of this enzyme in extracts of cells of a multiple aromatic auxotroph grown on limiting shikimic acid (BROWN and DOY 1963). It should be noted, however, that MITSUHASHI and DAVIS (1954) reported a 10-fold increase in the concentration of 5-dehydroquinase in extracts of certain mutants, namely, *Aerobacter* A170-143S1 selected for good growth response to quinic acid or 5-dehydroquinic acid, and *E. coli* 83-2 which accumulates dehydroshikimic acid. It is possible therefore, that in the experiments reported here and earlier (BROWN and DOY 1963) the growth conditions were not optimal for derepression of this enzyme.

The repression studies on chorismate mutase are difficult to interpret since the two components of this activity were not separated. However, it is possible to see that phenylalanine, in particular, has a marked repressing effect on total chorismate mutase activity. This effect has not previously been noted although COTTON and GIBSON (1965) did observe slight repression of chorismate mutase P by exogenous phenylalanine in wild-type *Aerobacter aerogenes*. Failure to observe significant repression by exogenous phenylalanine was probably due to the fact that this strain is prototrophic for phenylalanine. The fact that tryptophan does not repress overall chorismate mutase activity makes it unlikely that either of the two chorismate mutases (P and T) are repressed in coordination with the phenylalanine-inhibited DAHP synthetase which is multivalently repressed by phenylalanine and tryptophan.

Evidence has been presented for two unlinked genes (*aro G* and *aro F*) affecting the activity of the phenylalanine-inhibited and tyrosine-inhibited DAHP synthetases. This evidence does not indicate whether the blocks in DAHP synthetase activity in *aro G*⁻ and *aro F*⁻ strains are caused by alterations in the primary structures of the DAHP synthetases or by alterations in regulatory genes controlling the function of the respective structural genes.

The gene specifying the phenylalanine-inhibited DAHP synthetase (*aro G*) is unlinked to any other known gene concerned with phenylalanine biosynthesis. In contrast, the gene specifying the tyrosine-inhibited DAHP synthetase, *aro F*, is closely linked to the tyrosine gene (*tyr A*). This finding, and the observation that both the tyrosine-inhibited DAHP synthetase and chorismate mutase are

repressed by tyrosine raises the possibility of a tyrosine operon, regulated by tyrosine and containing the *aro F* and *tyr A* genes.

The failure of mutations in the *aro F* and *aro G* genes to eliminate the tryptophan-repressed DAHP synthetase suggested that this enzyme may be specified by a gene other than *aro F* or *aro G*. A direct test of this possibility could not be made as mutants altered in the tryptophan-repressed DAHP synthetase were not available. WALLACE and PITTARD (1967a), however, were able to isolate such mutants and have shown that the gene specifying the tryptophan-repressed DAHP synthetase (*aro H*) is separate from *aro F* and *aro G* and is closely linked to *aro D*, the gene specifying 5-hydroquinase. These workers also independently isolated and characterized mutants lacking the tyrosine-inhibited and phenylalanine-inhibited DAHP synthetases (PITTARD and WALLACE 1966; WALLACE and PITTARD 1967a, b).

SPRINSON and coworkers (personal communication) have also obtained mutants of *Salmonella typhimurium* which have analogous nutritional and biochemical properties to the *aro G*⁻ and *aro F*⁻ mutants described here. These findings and the biochemical evidence previously reported (GOLLUB, ZALKIN and SPRINSON 1966) suggest that the properties of DAHP synthetase in *S. typhimurium* may be closely similar to those in *E. coli*. In contrast, single-step mutants of *Bacillus subtilis* have been isolated in which all DAHP synthetase activity is lost (JENSEN and NESTER 1966). This finding, added to a considerable body of biochemical evidence, supports the view of JENSEN and NESTER that DAHP synthetase is a single enzyme in this organism.

Evidence that the lysine-inhibited and threonine-inhibited aspartate kinases are specified by two separate unlinked genes in *E. coli* has been obtained by PATTE and COHEN (1965) and COHEN, PATTE and TRUFFA-BACHI (1965). Thus, it would appear that, in *E. coli* and *Salmonella*, many of the mechanisms evolved for regulating aromatic biosynthesis are analogous to those evolved for the branched aspartate pathway.

The failure of tryptophan to repress tryptophan synthetase and the tryptophan-repressed DAHP synthetase in *trp R*⁻ strains shows that the product of the *trp R* gene is required for repression of both of these enzymes. This observation and that of ITO and CRAWFORD (1965) who showed that the *trp R* gene-product is required for repression of the whole tryptophan operon indicates that the tryptophan operon and *aro H* (the gene specifying the tryptophan-repressed DAHP synthetase) constitute a single physiological unit or regulon. The term, regulon, was coined by MAAS and CLARK (1964) to describe a group of genes controlling related functions and regulated together by the same repressor substance. Two types of regulons were distinguished; those in which the structural genes for the enzymes are adjacent to each other, for example, the histidine pathway, and which thus consist of single operons, and those in which they are not, for example, the arginine pathway, and which thus consist of several operons. It is proposed here that the tryptophan regulon is of the latter (arginine) type since it consists of two unlinked operons, one specifying the tryptophan pathway

enzymes, the other the tryptophan-repressed DAHP synthetase, both regulated by a repressor substance specified, at least in part, by the *trp R* gene.

It is not yet known whether the mutation to *trp R*⁻ affects the formation of tryptophanyl-t-RNA or whether it causes an alteration in an unknown, hypothetical repressor substance. In view of the findings with *trp R*⁻ strains it would be interesting to know whether the *trp S* mutations of HIRAGA *et al.* which map near the *str* locus on the *E. coli* chromosome and which restrict the derepression of the tryptophan operon, similarly affect the repressibility of the tryptophan-repressed DAHP synthetase.

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SUMMARY

Repression by the aromatic amino acids of several enzymes of aromatic biosynthesis in *Escherichia coli* K12 is reported. Repression of the phenylalanine-inhibited 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthetase is multivalent, requiring both phenylalanine and tryptophan. The tyrosine-inhibited DAHP synthetase is repressed by low concentrations of tyrosine and by high concentrations of phenylalanine and tryptophan. The third DAHP synthetase is repressed by tryptophan. This repression is not coordinate with the repression, by tryptophan, of tryptophan synthetase. Little repression of 5-dehydroquinase synthetase and 5-dehydroquinase was detected. Total chorismate mutase activity (consisting of chorismate mutases P and T) is repressed by phenylalanine and tyrosine. — Two classes of mutants, each lacking one of the three DAHP synthetases, were isolated and characterized. The gene specifying the phenylalanine-inhibited DAHP synthetase (*aro G*) is 90% cotransducible with a gene of the galactose region and 85% cotransducible with a gene controlling immunity to colicin E (*cim*). The gene specifying the tyrosine-inhibited DAHP synthetase (*aro F*) is 65% cotransducible with the *tyr A* gene and 50% cotransducible with the *phe A* gene. — The tryptophan-repressed DAHP synthetase could not be repressed by tryptophan in strains mutated in the tryptophan regulatory gene, *trp R*. Such strains are also constitutive for the tryptophan pathway enzymes.

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