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Genetic and Biochemical Analysis of the Isoenzymes Concerned in the First Reaction of Aromatic Biosynthesis in *Escherichia coli*

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ABSTRACT

Mutant strains of *Escherichia coli* K-12 were isolated possessing mutations which affected the tyrosine-inhibitable 3-deoxy-D-arabinoheptulosonic acid-7-phosphate (DAHP) synthetase, the phenylalanine-inhibitable DAHP synthetase, or the tryptophan-repressible DAHP synthetase. The mutations causing the loss of each of these activities have been mapped and are widely separated from each other on the *E. coli* chromosome. Chromatography on diethylaminoethyl cellulose columns allowed the recognition of four peaks of activity.

In a previous communication describing the distribution and function of a number of genes concerned with aromatic biosynthesis in *Escherichia coli* K-12 (19), we briefly reported on the isolation of a mutant strain of *E. coli* which, although able to grow on minimal medium, had a strict requirement for tyrosine in the presence of phenylalanine and tryptophan. This growth inhibition by phenylalanine and tryptophan could also be reversed by dehydroquinate, and it was suggested that this mutant had lost one of the three isoenzymes involved in the conversion of erythrose-4-phosphate and phosphoenolpyruvate to 3-deoxy-D-arabinoheptulosonic acid-7-phosphate (DAHP). Work from a number of other laboratories (5, 6, 11, 20) had indicated that in *E. coli* strains W and K-12, this reaction could be carried out by three isoenzymes, the formation of one of which was repressed by phenylalanine, one by tyrosine, and one by tryptophan. In addition to repression, the activity of the phenylalanine-repressible isoenzyme [DAHP synthetase (phe)] was inhibited by phenylalanine, and the activity of the tyrosine-repressible isoenzyme [DAHP synthetase (tyr)] was inhibited by tyrosine. The activity of the tryptophan-repressible isoenzyme [DAHP synthetase (trp)] was, on the other hand, not inhibited by tryptophan. We had suggested that the mutation in the strain unable to grow in the presence of phenylalanine and tryptophan affected the DAHP synthetase (tyr) isoenzyme. It is the purpose of this communication to provide further biochemical evidence in support of this interpretation and to describe the isolation and characterisation of mutant strains lacking DAHP synthetase (phe) and DAHP synthetase (trp).

Previous studies on the DAHP synthetase isoenzymes in *E. coli* have depended on ammonium sulfate fractionations to separate different activities (6, 20). Although this work has led directly to the conclusion that there are three isoenzymes, this procedure has not allowed the complete separation of DAHP synthetase (trp) and DAHP synthetase (tyr) and has relied to a large extent on repression and inhibition of particular isoenzymes to assist in their identification. In the present work, cell-free extracts were chromatographed on diethylaminoethyl (DEAE) cellulose, and evidence for the existence of at least three and possibly four DAHP synthetase isoenzymes was obtained.

In addition, genetic evidence will be presented describing the mapping of each of these genes: *aroF*, the structural gene for DAHP synthetase (tyr); *aroG*, the structural gene for DAHP synthetase (phe); and *aroH*, the structural gene for DAHP synthetase (trp). Genes *aroG* and *aroH* have not previously been described, and we have given them gene designations according to the principles presented in a previous paper (19).

MATERIALS AND METHODS

Organisms. The strains used in this work (Table 1) are all derivatives of *E. coli* K-12. The order of transfer of chromosomal genes by the Hfr strains is shown in Table 2.

Virulent λ phage was obtained from B. Holloway.

Media and culture methods. The media and culture

TABLE 1. *List of strains*[a]

Strain no.	Genetic loci relevant to this work						Sex
	aroB	*aroD*	*aroF*	*aroG*	*aroH*	Other loci	
AB2825	+	+	+	+	+	*thi⁻ his⁻ pro⁻ arg⁻ ilv⁻*	F⁻
AB2895	+	+	+	+	+	*thi⁻ his⁻ pro⁻ arg⁻ ilv⁻ mal⁻*	F⁻
AB3241	351	+	+	+	+	*thi⁻ his⁻ pro⁻ arg⁻ ilv⁻*	F⁻
AB2874	+	+	363	+	+	*thi⁻ his⁻ pro⁻ arg⁻ ilv⁻*	F⁻
AB2893	+	+	363	+	+	*thi⁻ his⁻ pro⁻ arg⁻ ilv⁻ mal⁻*	F⁻
AB3242	351	+	363	+	+	*thi⁻ his⁻ pro⁻ arg⁻ ilv⁻*	F⁻
AB2891	+	+	363	365	+	*thi⁻ his⁻ pro⁻ arg⁻ ilv⁻ T6-r*	F⁻
AB3244	+	+	363	365	+	*thi⁻ his⁻ pro⁻ arg⁻ ilv⁻ mal⁻*	F⁻
AB3245	351	+	363	365	+	*thi⁻ his⁻ pro⁻ arg⁻ ilv⁻*	F⁻
AB3248	+	+	363	365	367	*thi⁻ his⁻ pro⁻ arg⁻ ilv⁻ T6-r*	F⁻
AB1360	+	362	+	+	+	*thi⁻ his⁻ pro⁻ arg⁻*	F⁻
AB3254	+	+	+	+	367	*thi⁻ his⁻ pro⁻ arg⁻*	F⁻
AB3255	+	+	+	+	367	*thi⁻ his⁻ pro⁻ arg⁻ mal⁻*	F⁻
AB3256	351	+	+	+	367	*thi⁻ his⁻ pro⁻ arg⁻*	F⁻
AB2875	+	+	364	+	+	*thi⁻ his⁻ pro⁻ arg⁻ ilv⁻*	F⁻
AB2892	+	+	363	366	+	*thi⁻ his⁻ pro⁻ arg⁻ ilv⁻*	F⁻
AB3246	+	+	363	366	+	*thi⁻ his⁻ pro⁻ arg⁻ ilv⁻ mal⁻*	F⁻
AB3247	351	+	363	366	+	*thi⁻ his⁻ pro⁻ arg⁻ ilv⁻ mal⁻*	F⁻
AB3249	+	+	363	365	368	*thi⁻ his⁻ pro⁻ arg⁻ ilv⁻*	F⁻
AT2092	+	+	+	+	+	*theA⁻*	F⁻
AT2273	+	+	+	+	+	*tryA⁻*	F⁻
AB313	+	+	+	+	+	*thr⁻ leu⁻ thi⁻ T6-s*	♂ Hfr
AB311	+	+	+	+	+	*thr⁻ leu⁻ thi⁻ T6-s*	♂ Hfr
AB259	+	+	+	+	+	*thi⁻ str-s*	♂ Hfr
AB3243	+	+	363	365	+	*thi⁻ his⁻ pro⁻ arg⁻ ilv⁻ str-r*	F⁻
AB3250	+	+	+	365	+	*thi⁻ his⁻ pro⁻ arg⁻ ilv⁻*	F⁻
AB2826	351	+	+	+	+	*thi⁻*	F⁻

[a] The following abbreviations are used: *aro*, aromatic amino acids and vitamins; *pro*, proline; *pur*, purine; *thi*, thiamine; *arg*, arginine; *his*, histidine; *thr*, threonine; *leu*, leucine; *ilv*, isoleucine and valine; *phe*, phenylalanine; *tyr*, tyrosine; *trp*, tryptophan; *mal*, maltose; *xyl*, xylose; *str*, streptomycin; *T6*, bacteriophage T6; *s*, sensitive; *r*, resistant; (O), origin; *aroB*, structural gene for dehydroquinate synthetase; *aroD*, structural gene for dehydroquinase; *aroF*, structural gene for DAHP synthetase (tyr); *aroG*, structural gene for DAHP synthetase (phe); *aroH*, structural gene for DAHP synthetase (trp).

TABLE 2. *Order of transfer of chromosomal genes by Hfr strains*[a]

Strain no.	Order of transfer
AB313	(O) *xyl-mal-str-purC-his-trp-pro-leu-ilv-sex factor*
AB311	(O) *his-try-pro-leu-ilv-xyl-mal-str-purC-sex factor*
AB259	(O) *leu-pro-trp-his-purC-str-mal-xyl-ilv-sex factor*

[a] For abbreviations, see footnote to Table 1.

methods used in this work were described by Adelberg and Burns (1).

Buffers. The tris(hydroxymethyl)aminomethane (Tris) chloride and sodium phosphate buffers used were prepared by the method of Dawson and Elliott (10).

Chemicals. The chemicals used were obtained commercially and not further purified. Reagent grade Selectacel (DEAE cellulose), obtained from the Brown Co., Berlin, N.H., was used for chromatography. D-Erythrose-4-phosphate dimethylacetal dicyclohexylammonium salt (A grade) was obtained from Calbiochem, Los Angeles, Calif. Free D-erythrose-4-phosphate was prepared by the method of Ballou, Fischer, and MacDonald (4). A sample of DAHP (barium salt) was obtained from D. B. Sprinson. A filtered supernatant fluid containing accumulated products of *E. coli* 83-1 (22) was used as a source of dehydroquinic acid for nutritional tests.

Mating Procedures. The conditions under which the conjugation experiments were carried out have been described (19).

Transduction. Transductions involving phage P1 were carried out as previously described (19). Transductions involving phage λ, with either high frequency of transduction (HFT) or low frequency of transduction (LFT) lysates were carried out as described by Morse, Lederberg, and Lederberg (16). In measuring cotransduction frequencies, at least 100 transductants were tested for unselected markers.

Isolation of mutants. The conditions under which

cells were treated with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine were those described by Adelberg, Mandel, and Chen (2). The isolation of mutants from treated cultures involved a modification of either the penicillin enrichment technique described by Adelberg and Meyers (3) or the procedure of delayed enrichment described by Lederberg and Tatum (14). In both cases phenotypic expression of the mutants was allowed to occur by growth in complete medium for 2 hr after treatment with the mutagen.

Growth of cells and preparation of cell-free extracts. Cells were grown in minimal medium supplemented with the required nonaromatic amino acids, 1.2×10^{-4} M shikimic acid and 0.005% yeast extract. Cells harvested from 18- to 20-hr cultures were washed with chilled 0.9% NaCl and then suspended in 0.1 M Tris (*p*H 7.8). Cells were smashed by being forced through a French press at a pressure of 20,000 psi. Cell-free extracts, obtained by centrifugation at $21,600 \times g$ for 20 min, were dialyzed for 2 hr at 2 to 4 C against 0.01 M phosphate buffer containing 10^{-3} M mercaptoethanol.

Column chromatography of enzymes. Chromatography was carried out by the method described by Cotton and Gibson (9) except that the DEAE cellulose column was treated by a linear gradient elution starting with 500 ml of 0.01 M phosphate buffer (*p*H 7.0) in the mixing bottle and 500 ml of the same buffer containing 0.5 M NaCl in the inlet bottle. The eluent was passed through the column at the rate of 150 ml/hr by means of an LKB-minipump, and fractions were collected every 3.4 min, i.e., 8.4-ml fractions were collected. Nucleic acids were not removed prior to addition of cell-free extracts to the column.

Assay of DAHP synthetase. The method described by Brown and Doy (6) was used.

Protein estimation. Protein was estimated by the method of Lowry et al. (15).

Dehydroquinate synthetase assay. The method described by Srinivasan and Sprinson (21) was used.

Inhibition tests. Tests were carried out as described for the assay of DAHP synthetase with and without the amino acids and vitamins as indicated. The concentration of inhibitor was 10^{-3} M. Results are expressed as percentage inhibition of test compared with control without added inhibitor.

RESULTS

Isolation of DAHP synthetase mutants. The known pattern of end-product inhibition and repression of the three DAHP synthetase isoenzymes indicated a relatively simple procedure for isolating these mutants. Mutant strains lacking the tyrosine-sensitive isoenzyme should be unable to grow on minimal medium supplemented with phenylalanine and tryptophan but should grow on minimal medium alone or on minimal medium supplemented with phenylalanine, tyrosine, and tryptophan. By a strictly analogous argument, strains lacking the DAHP synthetase (phe) would not be expected to grow on minimal medium supplemented with tyrosine and tryptophan, and strains lacking DAHP synthetase (trp) should not grow on minimal medium supplemented with phenylalanine and tyrosine.

The parent strain used in this mutant isolation was a polyauxotrophic female, AB2825. From this strain, by the procedure outlined in Materials and Methods, two mutant strains were isolated with the expected properties of strains lacking DAHP synthetase (tyr). One of these mutants, strain AB2874, was again treated with mutagen, and two more strains were isolated, each with the expected properties of strains lacking both DAHP synthetase (tyr) and DAHP synthetase (phe); i.e., they could grow in minimal medium alone but could not grow on minimal medium supplemented with L-tryptophan, unless tyrosine and phenylalanine were also added.

One of these mutant strains, strain AB2891, was treated with mutagen once more, and strains were isolated that required all the aromatic amino acids for growth. The aromatic amino acid requirement of two of these mutants could be satisfied by dehydroquinate, and an enzyme analysis of crude cell-free extracts of these strains showed that they were completely unable to convert erythrose-4-phosphate and phosphoenolpyruvate to DAHP. The nutritional responses of these DAHP synthetase mutants and of a variety of recombinants obtained from them is shown in Table 3.

Genetic analysis of mutants. The approximate position on the bacterial chromosome of genes *aroF*, *aroG*, and *aroH* were obtained when mutant strains were crossed in uninterrupted matings with different Hfr strains and selection was made for the wild-type alleles of *mal*, *his*, *pro*, *ilv*, and *aroF*, *aroG*, or *aroH*. These approximate positions were confirmed by interrupted-mating experiments and by transduction experiments.

Map location of aroF. The gene *aroF*, whose approximate map position was somewhere between *mal* and *his*, was found to be cotransduced by phage P1 with either *pheA* or *tyrA*, with frequencies of 50 and 60%, respectively. In these experiments, P1 lysates were prepared on strain AB2874 (*aroF*$^-$) and used to transduce *pheA*$^+$ into AT2092 and *tyrA*$^+$ into AT2273.

These transductants were then tested for their ability to grow on minimal medium supplemented with phenylalanine and tryptophan; 50% of the *pheA*$^+$ transductants and 60% of the *tyrA*$^+$ transductants failed to grow.

Map location of aroG. The approximate map location of *aroG* was somewhere between *his* and *pro*, and a time-of-entry experiment carried out with Hfr AB259 gave a time of entry of 23 min for *aroG* compared with 14 min for *pro*. Since the genes concerned with galactose catabolism (*gal*)

were also transferred at about 23 min by Hfr AB259, a transduction experiment was carried out to determine whether *aroG* and *gal* were cotransducible by phage P1. Phage P1 prepared on AB259 (*aroG*$^+$ *gal*$^+$) was used to transduce *gal*$^+$ into strain AB3250 (*aroG*$^-$ *gal*$^-$).

The *gal*$^+$ transductants were selected on minimal medium containing galactose as sole carbon source and were then tested for their ability to grow on this medium supplemented with tyrosine and tryptophan. Of these transductants, 70% grew in the presence of tyrosine and tryptophan, indicating that they had integrated both *gal*$^+$ and *aroG*$^+$.

The specialized transducing phage λ can also transduce *gal*, and an experiment was carried out to determine whether λ could transduce *aroG* as well. A prototrophic *gal*$^+$ λ-lysogenic strain of *E. coli* K-12, AB264, was induced with ultraviolet light. The λ lysate (LFT) so obtained was used with strain AB2891 (*aroG*$^-$ *gal*$^-$ λ-lysogenic) as described in Materials and Methods, and selection was made for *gal*$^+$ transductants and for *gal*$^+$ *aroG*$^+$ transductants. Transductants having received *gal*$^+$ but not *aroG*$^+$ were obtained at a frequency of 5×10^{-7} plaque-forming units, whereas *gal*$^+$ *aroG*$^+$ transductants were obtained at the lower frequency of 5×10^{-8} per plaque-forming unit. A number of these *gal*$^+$ *aroG*$^+$ tranductants were purified and once more induced with ultraviolet light. Three of the eight transductants that were tested now produced HFT lysates which transferred *aroG*$^+$ *gal*$^+$ at a frequency of 10^{-2} per plaque-forming unit. One of these clones was studied for segregation of these markers, and *gal*$^-$ segregants were obtained that were also *aroG*$^-$.

Map location of aroH. The approximate position of *aroH* was determined to be somewhere between *his* and *pro*, and an interrupted-mating experiment with Hfr AB311 gave a time of entry for *aroH*$^+$ of 14 min compared with 6 min for *his*$^+$. Since this time of entry was identical with the time of entry of the *aroD*$^+$ gene, a transduction experiment was carried out to verify this close linkage. Phage P1 grown on strain AB3248 (*aroH*$^-$ *aroD*$^+$) was used to transduce *aroD*$^+$ into strain AB1360 (*aroD*$^-$ *aroH*$^+$). Transductants that had received *aroD*$^+$ were then tested for their ability to grow in the presence of phenylalanine and tyrosine; 68% failed to grow on minimal medium supplemented with phenylalaine and tyrosine, indicating 68% cotransduction of *aroH* and *aroD*. The relative positions of genes *aroF*, *aroG*, and *aroH* on the *E. coli* chromosome are shown in Fig. 1.

Biochemical analysis of parent and mutant strains. To compare the activities present in each of the mutant strains, it was necessary to standardize growth conditions; to increase specific activities, it was necessary to find conditions under which cells could be harvested in a derepressed condition. To do this, a second mutation was introduced into all of the mutant strains except AB3248, blocking aromatic biosynthesis at the second reaction involving the enzyme dehydroquinate synthetase. As a result of this, all strains then had an absolute requirement for all the aromatic amino acids and vitamins which could be satisfied by shikimic acid. For the preparation of cell-free extracts, cells were grown in minimal medium containing a growth-limiting concentration of shikimic acid (1.2×10^{-4} M). The mutation affecting dehydroquinate synthetase was introduced into these strains in the following manner. Derivatives of the parent and mutant strains which were unable to ferment maltose were obtained by selecting for resistance to virulent λ phage (13). Phage P1 prepared on strain AB2826 (*mal*$^+$ *aroB*$^-$) was then used to transduce *mal*$^+$ into the *mal*$^-$ derivatives; *mal*$^+$ transductants were selected on minimal medium containing maltose as the sole carbon source and

TABLE 3. *Growth responses of mutant strains*

Strain no.	Genotype	Growth response on minimal medium supplemented with					
		nil[a]	PHE TRP	TYR TRP	PHE TYR	TRP	PHE TYR TRP PAB POB
AB2825	*aroF*$^+$ *aroG*$^+$ *aroH*$^+$	+	+	+	+	+	+
AB2874	*aroF*$^-$ *aroG*$^+$ *aroH*$^+$	+	−	+	+	+	+
AB2891	*aroF*$^-$ *aroG*$^-$ *aroH*$^+$	+	−	−	+	−	+
AB3248	*aroF*$^-$ *aroG*$^-$ *aroH*$^-$	−	−	−	−	−	+
AB3250	*aroF*$^+$ *aroG*$^-$ *aroH*$^+$	+	+	−	+	+	+
AB3254	*aroF*$^+$ *aroG*$^+$ *aroH*$^-$	+	+	+	−	+	+

[a] Abbreviations used: nil, no supplement; PHE, phenylalanine; TYR, tyrosine; TRP, tryptophan; PAB, *p*-aminobenzoic acid; POB, *p*-hydroxybenzoic acid; +, growth after 24 hr; −, no growth after 24 hr.

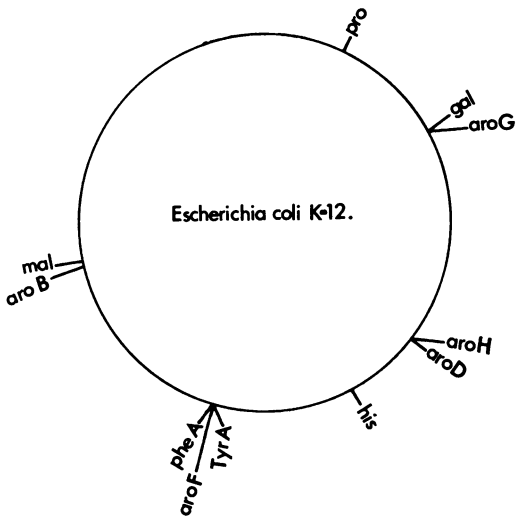

FIG. 1. *Genetic map of Escherichia coli showing the relative positions of aroF, aroG, and aroH. A description of the function of the genes shown is to be found in Table 1 and in the text. The relative order of pheA, aroF, tyrA, and aroD, aroH, and aroG, gal has not yet been determined.*

supplemented with phenylalanine, tyrosine, tryptophan, *p*-aminobenzoic acid, *p*-hydroxybenzoic acid, and shikimic acid. Transductants which were unable to grow on maltose minimal medium, i.e., *mal*$^+$ *aroB*$^-$, were purified and used for the biochemical analysis.

Cell-free extracts were prepared and chromatographed on DEAE cellulose as described in Materials and Methods. The resulting chromatographic patterns of enzyme activity are shown in Fig. 2, 3, and 4. Figure 2 shows the pattern of activities obtained when a cell-free extract of strain AB3241 was chromatographed. There are three separate peaks of activity, with the largest peak (fractions 48 to 64) appearing as if it is a composite of two overlapping peaks. That this is so was shown by inhibition studies in which the effect of either phenylalanine or tyrosine on the activity of these fractions was studied (Table 4). Whereas fractions 48 to 50 were predominantly tyrosine-sensitive (67% inhibition by tyrosine and only 23% by phenylalanine), fractions 54 to 60 showed an enrichment for the phenylalanine-sensitive activity (57% inhibition by phenylalanine and only 34% by tyrosine). The fact that the activities in the first two peaks were not inhibited by phenylalanine, tyrosine, or tryptophan, or by combinations of these, indicates that either one or both of these peaks represent the DAHP synthetase (trp) activity (6). Whereas both of these peaks were stable, losing no activity after heating at 45 C for 20 min or storage at −15 C for 28 days, the composite peak was much more unstable, losing 70% of its activity after storage at −15 C for 2 days. The presence of dithiothreitol (10^{-3} M) in the reaction mixture enhanced the activities of these unstable fractions, and this was therefore routinely added in assays from tube 40 onwards. This high level of instability may explain the low recoveries of activity (25 to 45%) after chromatography.

Figure 3a shows the pattern of activities obtained when a cell-free extract of strain AB3242 (*aroF*$^-$ *aroB*$^-$) was chromatographed. It will be remembered that this strain shows a requirement for tyrosine in the presence of phenylalanine and tryptophan and was thought to lack DAHP synthetase (tyr). It can be seen in Fig. 3a that the third peak is no longer a composite peak, and, although in this column peak fractions are coming out slightly earlier than in the previous column, a comparison of the figures suggests the loss of the first shoulder of the composite peak in the case of AB3242. This is the peak that was predominantly

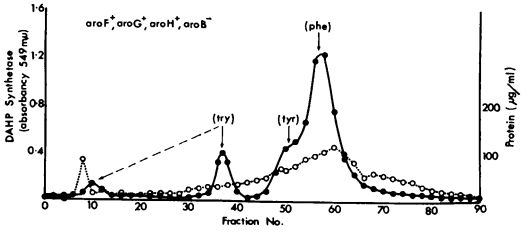

FIG. 2. *Chromatography of a cell-free extract of parent strain AB3241 (aro B*$^-$*, aroF*$^+$*, aroG*$^+$*, aroH*$^+$*). (●) DAHP synthetase activity; (○) protein. Symbols used: (phe), DAHP synthetase (phe); (tyr), DAHP synthetase (tyr); (try), DAHP synthetase (trp).*

TABLE 4. *Inhibition*[a] *by aromatic amino acids of fractions obtained from chromatography of a cell-free extract of wild-type strain AB3241*

Inhibitor (final concn, 10^{-3} M)	Fraction no.			
	8–10	36–38	48–50	54–60
L-Phenylalanine	3	0	23	57
L-Tyrosine	1	0	67	34
L-Tryptophan	1	0	—[c]	—
L-Phenylalanine plus L-tyrosine	—	—	68	80
Multiple supplement[b]	0	1.2	—	—

[a] Expressed as percentages.

[b] Consisted of L-phenylalanine, L-tyrosine, L-tryptophan, *p*-aminobenzoic acid, and *p*-hydroxybenzoic acid.

[c] Not tested.

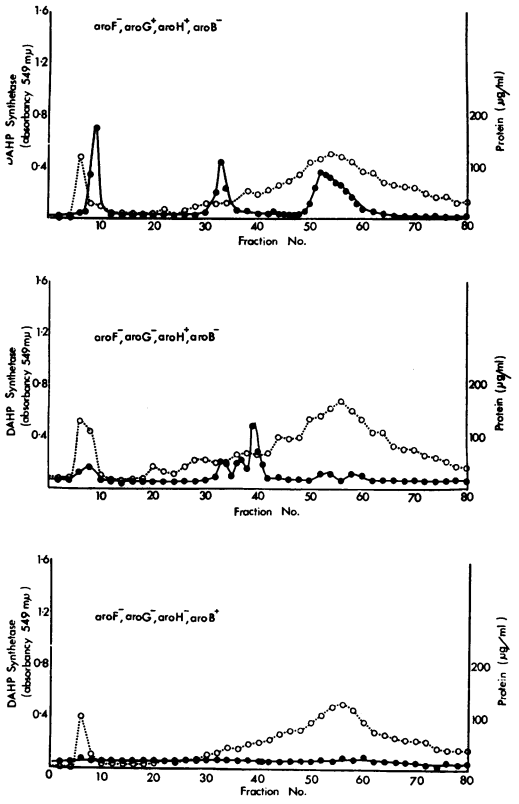

FIG. 3. *Chromatography of cell-free extracts of mutant strains. (a) Strain AB3242 (aroB⁻, aroF⁻ aroG⁺ aroH⁺); (b) strain AB3245 (aroB⁻ aroF⁻ aroG⁻ aroH⁺) (c) strain AB3248 (aroB⁺ aroF⁻ aroG⁻ aroH⁻). (●) DAHP synthetase activity; (○) protein.*

tyrosine-sensitive, and its loss is confirmed by the results of inhibition studies carried out on fractions 50 to 56 from the AB3242 column. The activity of these fractions is now predominantly phenylalanine-sensitive (94% inhibition), whereas tyrosine causes only a 15% inhibition, confirming the identity of the *aroF⁻* mutation as affecting the tyrosine-sensitive isoenzyme, DAHP synthetase (tyr).

Figure 3b shows the pattern of activities obtained when a cell-free extract of strain AB3245 (*aroF⁻ aroG⁻ aroB⁻*) was chromatographed. The first two peaks are still present, although diminished in size. Both the phenylalanine- and tyrosine-sensitive peaks are missing, but two new peaks of activity have appeared between fractions 36 and 41. These new peaks are very unstable and the activity decays rapidly; all activity disappeared after 6 hr at 0 to 2 C. Inhibition studies on fraction 39 show that it is not inhibited by either phenylalanine or tyrosine. The loss of the phenylalanine-sensitive peak confirms that the mutations in the *aroG* gene affect the phenylalanine-sensitive isoenzyme. The fact that strain AB3245 is unable to grow on minimal medium in the presence of tryptophan suggests that these new unstable peaks of activity must also be either repressible or inhibitable by tryptophan.

Figure 3c shows the pattern of activities obtained when a cell-free extract of strain AB3248 (*aroF⁻ aroG⁻ aroH⁻*) was chromatographed. The first two stable peaks and the new unstable peaks observed in the last column are all missing, as might be expected from the inability of strain AB3248 to grow on minimal medium alone and as was indicated by the failure to find any DAHP synthetase activity in the crude cell-free extract of this strain.

To investigate whether a mutation in the *aroH* gene can by itself cause the loss of each of the two early stable peaks, a new recombinant strain was isolated that was *aroH⁻ aroF⁺ aroG⁺*. Phage P1 prepared on AB3248 was used to transduce *aroD⁺* into strain AB1360 (*aroD⁻ aroF⁺ aroG⁺ aroH⁺*), and a transductant was selected that had integrated *aroD⁺ aroH⁻*. As previously noted, these genes are cotransduced at frequencies approaching 70%. The *aroB⁻* mutation was then introduced into this strain as described previously, to produce strain AB3256 (*aroH⁻ aroF⁺ aroG⁺ aroB⁻*.

A cell-free extract of this strain was chromatographed and the results are shown in Fig. 4. As expected, the second peak of activity is missing, but unexpectedly the first peak is still present. Another difference in this column is to be seen in the general composition of the third mixed peak. More activity now appears in the earlier fractions (48 to 50) than in the later fractions (54 to 60), in direct contrast to the wild-type column (Fig. 2). In the wild-type column, fractions 48 to 50 were identified as containing predominantly DAHP synthetase (tyr), and fractions 54 to 60 contained mainly DAHP synthetase (phe). Inhibition studies confirmed that, in the case of this mutant, DAHP synthetase (tyr) is still predominantly

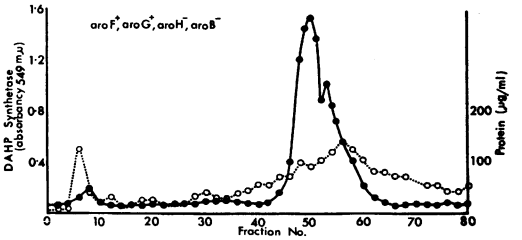

FIG. 4. *Chromatography of a cell-free extract of mutant strain AB3256 (aroB⁻ aroF⁺ aroG⁺ aroH⁻). (●) DAHP synthetase activity; (○) protein.*

found in fractions 48 to 50 and DAHP synthetase (phe) in fractions 54 to 60. For example, fraction 48 was inhibited 95% by tyrosine and only 15% by phenylalanine, whereas fraction 56 was inhibited 70% by phenylalanine and only 57% by tyrosine. This means, therefore, that the ratio of DAHP synthetase (phe) and DAHP synthetase (tyr) in the mutant has been significantly altered. Whether this alteration has been directly caused by the introduction of *aroH*$^-$ mutation or whether it reflects an alteration in enzyme levels due to a difference in the physiological state of cells has not yet been determined.

DISCUSSION

The chromatography of DAHP synthetase isoenzymes on DEAE cellulose and the isolation of mutants lacking different isoenzymes confirm and extend the earlier work on DAHP synthetase in *E. coli* (6, 20). A brief report by Brown and Maas (Federation Proc. **25**:338, 1966) has recently indicated that similar mutants of *E. coli* K-12 have been isolated in other laboratories.

The genes coding for the three major isoenzymes are widely separated on the chromosome of *E. coli*, and yet an analysis of the enzymes produced by the different mutants suggests that there may be some interaction among the various gene products. In particular, a mutation in the *aroG* gene has, as expected, been shown to cause the loss of the phenylalanine-sensitive isoenzyme; however, at the same time, one or more new unstable peaks of activity appeared, and the activities of the first two noninhibitable peaks decreased. Since tryptophan inhibits the growth of this mutant on minimal medium, it must be assumed, for the moment, that either the formation or activity of these stable and unstable noninhibitable fractions is controlled by tryptophan. A mutation in the *aroH* gene causes the loss of all the peaks of activity that are not inhibitable by phenylalanine or tyrosine, when it is introduced into a strain that is already *aroG*$^-$ *aroF*$^-$. On the other hand, a mutant strain with the genotype *aroG*$^+$ *aroF*$^+$ *aroH*$^-$ has lost only the second of the first two noninhibitable peaks.

It should be noted that the methods we used to obtain mutants with mutations in the *aroG* gene were such that any strains having lost both DAHP synthetase (phe) and DAHP synthetase (trp) as a result of a single mutation would not have been isolated. We are therefore currently investigating this possibility.

The possibility of enzyme aggregates has also been suggested by Brown and Doy (7), as a result of studies on *E. coli* W in which they observed examples of cross-repression. For example, they found that DAHP synthetase (phe) could be repressed by either phenylalanine or tryptophan.

From the results reported in this paper, it is not yet possible to decide whether there are in fact four isoenzymes or only three. There are certainly four peaks of activity, and it will require studies on enzyme repression to decide whether the formation of each of the first two peaks is repressed by tryptophan. If separate control mechanisms exist and it is concluded that there are four isoenzymes, then it will still have to be decided whether these are coded for by three or four genes. The only strain that we have examined which has lost the first peak of activity is strain AB3248 (*aroF*$^-$ *aroG*$^-$ *aroH*$^-$ *aroB*$^+$). This is unfortunately also the only strain that does not carry a mutation in the *aroB* gene, and this may affect the appearance of the first peak. For example, strain AB3248 grows very slowly on minimal medium containing tyrosine, phenylalanine, and tryptophan, whereas strains possessing the *aroB*$^-$ mutation do not grow at all unless either shikimic acid or *p*-aminobenzoic acid is added.

The separation of these isoenzymes on DEAE cellulose should greatly facilitate studies on repression and also studies concerned with the possible interaction of the gene products of *aroF*, *aroG*, and *aroH*.

The widely separated distribution of these genes on the chromosome (Fig. 1) makes it unlikely but not impossible that during evolution these multiple genes with related activities arose by duplication followed by mutation and selection for gene products sensitive to different control mechanisms. [A similar lack of clustering has been found in the case of the two genes coding for the two aspartate kinase enzymes of *E. coli* (8, 18).] This assumption is based on one observation of gene duplication, in which both genes were found to be next to each other (17), and on the fact that very few transpositions have been reported in *E. coli* (12). A more definite answer to this question, however, will depend on genetic and biochemical fine-structure analyses of these genes and their products. The significance of the grouping of *aroH* and *aroD* genes and *aroF* and *pheA* and *tyrA* is also still to be determined.

Finally, a new marker has been found that can be transduced by phage λ.

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