JOURNAL OF BACTERIOLOGY, Oct., 1966
Vol. 92, No. 4

Gene Controlling the Uptake of Shikimic Acid by *Escherichia coli*

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Received for publication 17 June 1966

ABSTRACT

PITTARD, JAMES (University of Melbourne, Melbourne, Australia), AND B. J. WALLACE. Gene controlling the uptake of shikimic acid by *Escherichia coli*. J. Bacteriol. 92:1070–1075. 1966.—Mutants possessing an impaired ability to take up exogenously supplied shikimic acid were isolated. Mutants showing either a complete or partial loss of this activity were found. The mutations have been mapped on the *Escherichia coli* chromosome and occur in a single gene, situated near to the cluster of genes concerned with histidine biosynthesis. The uptake of dehydroshikimic acid was also affected by these mutations, suggesting a common mechanism for the uptake of these two related intermediates of aromatic biosynthesis.

During the course of experiments investigating the distribution of genes concerned with aromatic biosynthesis in *Escherichia coli* K-12, further mutations were obtained in an aromatic auxotroph, strain AB1360, for the purpose of isolating shikimate kinase mutants. Strain AB1360 is unable to convert dehydroquinate to dehydroshikimate (see Fig. 1 for a description of the pathway), and consequently requires tyrosine, phenylalanine, tryptophan, and 4-aminobenzoic and 4-hydroxybenzoic acids for growth. These aromatic amino acids and vitamins can be replaced by either shikimic acid or dehydroshikimic acid, two intermediates of the common pathway of aromatic biosynthesis (Fig. 1). Strain AB1360 was treated with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and mutant strains were isolated whose aromatic requirements could no longer be satisfied by shikimic acid. In some cases, the mutants were able to grow in the presence of shikimic acid and tyrosine; in other cases, all of the aromatic amino acids were required for growth, whether shikimic acid was added to the medium or not. It was expected that these mutant strains would be blocked in one of the reactions converting shikimate to chorismate, or in the case of strains growing on shikimic acid plus tyrosine, one of the reactions converting chroismate to tyrosine. The results of biochemical and genetic analyses of two of these strains, however, indicate that the failure of shikimic acid to satisfy the aromatic requirements of these cells is not due to the loss of any of the enzymes involved in the conversion of shikimate to chorismate or chorismate to tyrosine, but is due to the inability of these cells to take up shikimic acid from the medium.

MATERIALS AND METHODS

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

Media and culture methods. The media and culture methods used in this work have been described by Adelberg and Burns (1).

Chemicals. Most of the chemicals used were obtained commercially and not further purified. Shikimic acid-C^{14} was obtained from New England Nuclear Corp., Boston, Mass. Dehydroshikimic acid was not available commercially; it was obtained from a mutant strain of *E. coli* blocked in the conversion of dehydroshikimate to shikimate (4). The dehydroshikimic acid was not purified but was concentrated from the accumulation supernatant fluid by extraction into ethyl acetate at a *p*H of 2, evaporated to dryness, and dissolved in phosphate buffer.

Nutritional tests. The methods used for testing the nutritional requirements of mutant strains have been described previously (4).

Mating conditions and interruption of mating. The conditions under which matings were carried out and interrupted have been described previously (4).

Transduction techniques. Transduction techniques used in this work have been described previously (4).

Isolation of mutants. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was used as mutagen; the procedure used was that described by Adelberg, Mandel, and Chen (2).

Preparation of cell-free extracts. The methods used to prepare cell-free extracts have been described previously (4).

Assay of enzymes involved in the conversion of

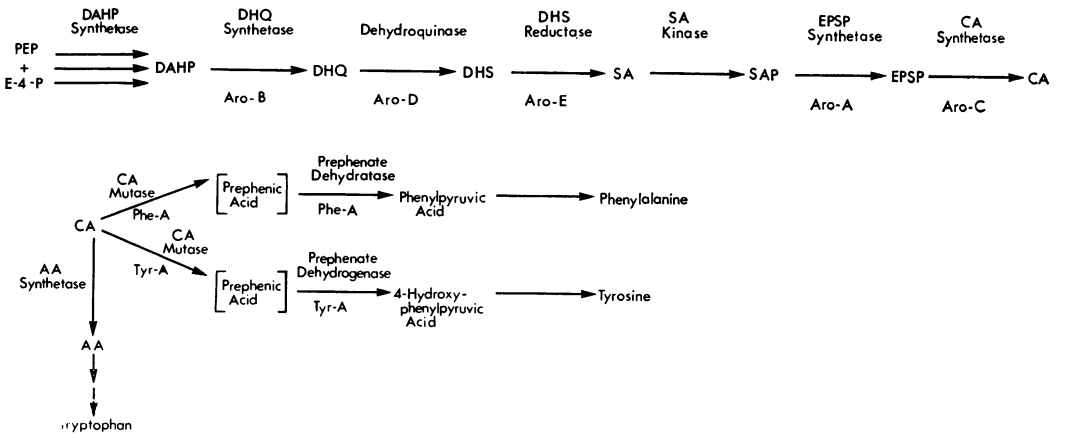

FIG. 1. *Outline of the reactions involved in the conversion of erythrose-4-phosphate and phosphoenolpyruvate to chorismate and of chorismate to phenylalanine and tyrosine. The genetic symbols for each of the known structural genes are also included. Symbols used: PEP, phosphoenolpyruvate; E-4-P, erythrose-4-phosphate; DAHP, 3-deoxy-D-arabino-heptulosonic acid-7-phosphate; DHQ, dehydroquinate; DHS, dehydroshikimate; SA, shikimate; SAP, shikimate-5-phosphate; EPSP, 3-enolpyruvylshikimate-5-phosphate; CA, chorismate; AA, anthranilate; Aro-B, the structural gene for DHQ synthetase; Aro-D, the structural gene for dehydroquinas, etc.*

TABLE 1. *List of strains*[a]

Strain	Auxotrophic characters and energy source utilization								Response to		Sex
	his	*pro*	*arg*	*aromD*	*shiA*	*thr*	*leu*	*xyl*	*T6*	*str*	
AB1360	−	−	−	362	+	+	+	−	*r*	*s*	♀
AB2879	−	−	−	362	351	+	+	−	*r*	*s*	♀
AB2905	−	−	−	362	351	+	+	−	*r*	*r*	♀
AB2880	−	−	−	362	352	+	+	−	*r*	*s*	♀
AB311	+	+	+	+	+	−	−	+	*s*	*r*	♂ Hfr
AB259	+	+	+	+	+	+	+	+	*s*	*s*	♂ Hfr
AB313	+	+	+	+	+	−	−	+	*s*	*r*	♂ Hfr

[a] Abbreviations: *his*, histidine; *pro*, proline; *arg*, arginine; *aro*, aromatic amino acids and vitamins; *shi*, shikimic acid; *thr*, threonine; *leu*, leucine; *xyl*, xylose; *T6*, bacteriophage T6; *str*, streptomycin; *r*, resistant; *s*, sensitive. Numbers refer to allele numbers that have been allotted to mutant loci in these laboratories; allele numbers are given only for the loci under consideration.

shikimate to tyrosine. The methods used to assay the various enzymes involved in the conversion of shikimate to *p*-hydroxyphenylpyruvate have been described previously (4).

The assay for transaminase activity was carried out by testing for the conversion of L-tyrosine to 4-hydroxyphenylpyruvate by use of the following system (Gibson, *personal communication*). The reaction mixture contained L-tyrosine, 0.50 μmole; α-keto-glutarate, 5.0 μmoles; pyridoxal phosphate, 0.06 μmole, magnesium chloride, 10 μmoles; tris(hydroxymethyl)-aminomethane chloride buffer (*p*H 8.2), 50 μmoles; and an appropriate amount of enzyme preparation, in a total volume of 1.0 ml. This was incubated for 30 min at 37 C, 3.0 ml of 0.1 N NaOH was then added, and the mixture incubated for a further 30 min at 37 C to allow for development of the chromophore. The optical density at 330 mμ was then read against a water blank in a Unicam SP500 spectrophotometer.

Preparation of cells for uptake experiments and the measurement of C^{14}-shikimate uptake. Cells were grown overnight in minimal medium supplemented with 10^{-4} M tyrosine, phenylalanine, and tryptophan, 10^{-5} M shikimic acid, and 10^{-6} M 4-hydroxybenzoic acid and 4-aminobenzoic acid. [The term minimal medium will be used to describe a half-strength preparation of medium 56 of Monod et al. (3), supplemented with glucose, thiamine, histidine, proline, and arginine.] Cultures were aerated by shaking on a New Brunswick Gyrotory shaker-bath. A small volume (0.3 ml) of the overnight culture was inoculated into 10 ml of the same medium and incubated with aeration until the cells achieved early exponential phase (about 4×10^8 cells per milliliter). At this stage, the cells were centrifuged, washed once with sterile buffer, and resuspended in minimal medium. These washed-cell suspensions contained approximately 2×10^8 cells per milliliter and were incubated

with aeration for 45 min to exhaust any endogenous store of shikimic acid. At zero-time, C^{14}-labeled shikimic acid was added to the flask to a final concentration of 10^{-5} M and a specific activity of 2×10^6 counts per min per μmole. During the period of the experiment, the cells were shaken gently at 37 C in a water bath. Samples (1.0 ml) were withdrawn at 5-min intervals and filtered on membrane filters (HA, 0.45-μ; Millipore Filter Corp., Bedford, Mass.) by use of a 10-ml syringe fitted with a Millipore Swinny adapter. The cells were washed with two successive 5-ml volumes of chilled phosphate buffer, and the filters were removed, allowed to dry, glued to planchets, and counted in a Nuclear Chicago automatic gas flow counting system.

RESULTS

After treatment of strain AB1360 with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, 12 strains were recovered whose growth requirement for aromatic amino acids was not satisfied by shikimic acid alone. Some of these strains grew when tyrosine was added to the shikimic acid, others required tyrosine and phenylalanine, and still others required tyrosine, phenylalanine, tryptophan, 4-aminobenzoic acid, and 4-hydroxybenzoic acid. When cell-free extracts of these strains were prepared, they all showed wild-type activity for the conversion of shikimate to shikimate-5-phosphate and for the conversion of shikimate to anthranilate. These results were supported by genetic evidence; transduction tests showed that none of these new mutations was linked to either the *aroC* or the *aroA* gene (see Fig. 1). Two of these mutant strains, AB2879 and AB2880, were chosen for further study. Strain AB2879 required all the aromatic amino acids and vitamins for growth, even in the presence of shikimic acid, whereas strain AB2880 required only shikimic acid and tyrosine. Cell-free extracts of both strains possessed approximately the same activity per milligram of protein for converting shikimate to anthranilate as did the parent strain AB1360. In addition, cell-free extracts of strains AB2880 and AB1360 were both chromatographed on diethylaminoethyl cellulose and examined for prephenate dehydrogenase and its associated chorismate mutase activity and also for transaminase activity. The results were the same in both cases, the extracts possessing full activities for all these reactions.

The mutation carried by strain AB1360 which affects aromatic biosynthesis occurs in the *aroD* gene (4). For reasons which will soon become apparent, and to simplify the discussion of results, we propose at this point that the extra mutations in strains AB2879 and AB2880 affecting shikimate utilization both occur in another gene, which we term *shiA*. Some of the evidence for considering that the mutations affecting both AB2879 and AB2880 occur in the same gene are as follows. Phage P1, which had been prepared on strain AB2880, was used in a transduction experiment, with strain AB2879 as the recipient. Transductants were selected on minimal medium supplemented with shikimate and tyrosine. These transductants were then tested for their ability to grow on minimal medium supplemented only with shikimate; 37 of 40 failed to grow in the absence of tyrosine. The simplest explanation for this result is that the mutations in strains AB2879 and AB2880 which cause an impaired utilization of shikimate are sufficiently close together on the chromosome so that the transducing particle which introduces the *shiA 351*$^+$ allele into the recipient cell (AB2879 *shiA 351*$^-$) also carries the *shiA 352*$^-$ allele of the donor (AB2880). As a result of this, most of the transductants integrate *shiA 351*$^+$ and *shiA 352*$^-$ and possess the genotype and phenotype of the donor organism. The high frequency with which this occurs (37 of 40) is compatible with the supposition that these two alleles occur in the same gene (4).

Map location of the shiA gene. The mutants AB2879 and AB2880 were crossed with the male strain, AB313, in an uninterrupted mating, and *xyl*$^+$, *his*$^+$, *aroD*$^+$, *shiA*$^+$, and *pro*$^+$ recombinants were selected. The points of origin and the direction of transfer of genes by the Hfr strains used in this work are shown in Fig. 2. In both crosses, absolute numbers of recombinants for *his*$^+$,

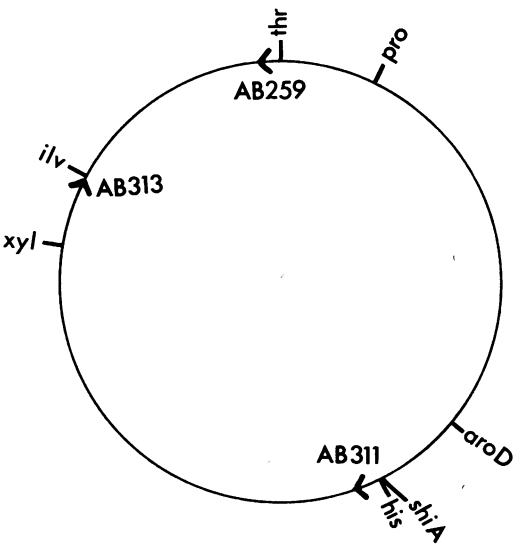

FIG. 2. *Map of the Escherichia coli chromosome showing the points of origin and direction of transfer of three different Hfr strains and the relative position of the genetic markers relevant to this work.*

aroD$^+$, and *shiA*$^+$ were very similar, indicating that *shiA* was situated near to both *his* and *aroD*. To investigate whether the *shiA* and *aroD* genes were cotransducible, phage P1 which had been propagated on a wild-type strain was used with AB2880 as the recipient. Transductants were selected on minimal medium supplemented with tyrosine (*aroD*$^+$ selection) and on minimal medium supplemented with shikimate (*shiA*$^+$ selection). Transductants were then tested for their ability to grow on minimal medium alone. Of the *aroD*$^+$ transductants, 100% exhibited an *aroD*$^+$ *shiA*$^+$ phenotype and were able to grow on minimal medium alone. About 60% of the transductants selected on minimal medium plus shikimate were able to grow on minimal medium alone, and 40% still required shikimate. The simplest interpretation of these results was that the *aroD* gene and the *shiA* gene were themselves close enough on the *E. coli* chromosome to be cotransducible. When strain AB2879 was used as a recipient, again a high percentage (80%) of the transductants selected on minimal medium plus shikimate (*shiA*$^+$ selection) were subsequently able to grow on minimal medium alone. Although it was not possible in this case to select for the *aroD*$^+$ phenotype, transductants were also readily obtained on minimal medium (*shiA*$^+$ *aroD*$^+$ phenotype).

Interrupted-mating experiments. To confirm the very close linkage of the genes *aroD* and *shiA*, strains AB2879 and AB2880 were crossed with the Hfr AB311. Mating was interrupted at suitable intervals, and samples were plated to the necessary minimal media to select *his*$^+$, *shiA*$^+$, and *aroD*$^+$ *shiA*$^+$ recombinants. The results of the cross involving strain AB2879 as the recipient are shown in Fig. 3. As can be seen, the *shiA*$^+$ gene has an almost identical time of entry to that of the *his*$^+$ gene and is unexpectedly transferred approximately 5 min before the *aroD*$^+$ gene. The same results were obtained when strain AB2880 was used as the recipient. According to these results, *shiA* should be cotransducible with *his* but not with *aroD*. Phage P1 was then prepared on a wild-type strain and was used to transduce *his*$^+$ into both AB2879 and AB2880. These transductants were then tested for their inheritance of the *shiA*$^+$ gene. Approximately 70% of the *his*$^+$ transductants in both cases had received and integrated the *shiA*$^+$ gene. In other words, transduction experiments indicate that *shiA* is cotransducible with *his* and with *aroD*. Yet *his* and *aroD* are not cotransducible and can be shown in interrupted-mating experiments to be about 6 min apart (a distance at least three times larger than the size of the largest chromosomal fragment to be transduced by phage P1).

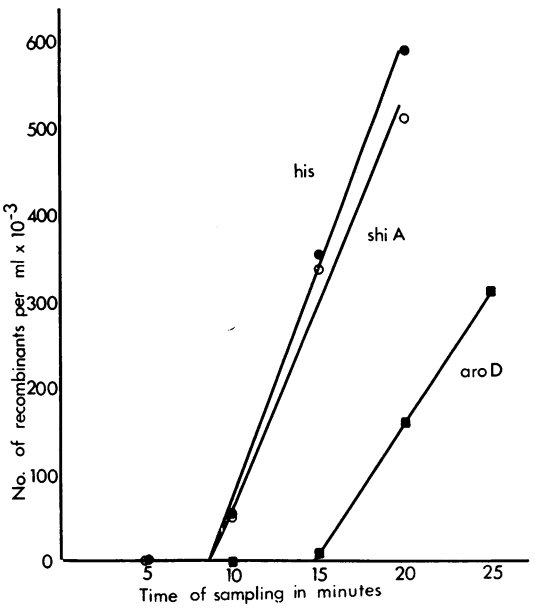

FIG. 3. *Kinetics of zygote formation when the Hfr AB311 was mated with the recipient AB2879. Identical curves were obtained with the recipient AB2880.*

This dilemma can be resolved if the results of the transduction in which both *shiA*$^+$ and *aroD*$^+$ appeared to be cotransduced are interpreted in a different manner. What we propose is that *shiA* and *aroD* are not cotransducible but that the effect of a *shiA*$^-$ mutation is expressed only in a cell which carries an *aroD*$^-$ mutation and which is growing on exogenously supplied shikimic acid. If, for example, the *shiA*$^-$ mutation affects the uptake of shikimic acid, then as soon as such a cell is converted to *aroD*$^+$ and is able to make shikimate endogenously the *shiA*$^-$ mutation will no longer be expressed as a requirement for extra growth factors. For this reason, although only 60% of the transductants selected on minimal medium plus shikimate can grow on minimal medium, 100% of the transductants selected on minimal medium plus tyrosine can grow on minimal medium. The latter transductants would all be *aroD*$^+$ *shiA*$^-$ whereas the former would be a mixture of *aroD*$^+$ *shiA*$^-$ and *aroD*$^-$ *shiA*$^+$. If this explanation is true, then it should be possible to produce revertants from strain AB2879 and strain AB2880 which can grow on minimal medium just as readily as such revertants are produced from strain AB1360. Also, if strains AB2880 and AB2879 are crossed with the Hfr AB259 which transfers *aroD*$^+$ before *his*$^+$, the time of entry for *shiA*$^+$ (selection on minimal medium plus shikimate) should appear to be identical

with that of *aroD*$^+$ and to precede *his*$^+$ by approximately 5 min. In other words, the cell's requirements for aromatic amino acids in addition to shikimate will disappear as soon as either the *aroD*$^+$ or the *shiA*$^+$ gene is transmitted to the recipient. When Hfr AB311 is the donor, the *shiA*$^+$ gene is transferred first, and it is possible to get distinct time-of-entry curves for both *shiA*$^+$ and *aroD*$^+$. When Hfr strain AB259 is used as the donor, however, the *aroD*$^+$ gene is the first to be transferred; when the cell has received *aroD*$^+$, it becomes phenotypically *aroD*$^+$ *shiA*$^+$, although it still retains the *shiA*$^-$ allele. That this latter prediction is true is shown by the time-of-entry curves in Fig. 4. (The recipient used in this cross, strain AB2905, is a streptomycin-resistant derivative of strain AB2879.)

No quantitative studies have been carried out on the reversion of AB2879, AB2880, and AB1360, but all strains when treated with the mutagen *N* - methyl - *N'* - nitro - *N* - nitrosoguanidine produce revertants able to grow on minimal medium.

Biochemical function of the shiA gene. To test whether the *shiA* gene was concerned with shikimic acid uptake, the uptake of C^{14}-labeled shikimic acid by the two mutant strains and the parent AB1360 was measured (Fig. 5). Compared with the parent strain AB1360, the rate of uptake of shikimic acid by strain AB2880 was reduced by approximately 40%, whereas strain AB2879 did not take up shikimic acid at all. It is to be remembered that strain AB2880 can grow on shikimate plus tyrosine, whereas strain AB2879 requires the five aromatic supplements. To examine further the hypothesis that the *shiA*$^-$ mutation was concerned only with the uptake of shikimic acid, *aroD*$^+$ transductants of strain AB1360 and of strain AB2879 were selected on minimal medium. These transductants would be expected to possess the genotypes *aroD*$^+$ *shiA*$^+$ and *aroD*$^+$ *shiA*$^-$, respectively. One of each of these transductants was purified and examined for its ability to take up shikimic acid. The *aroD*$^+$ *shiA*$^-$ transductant, which is able to grow in the absence of the aromatic amino acids, nevertheless did not take up shikimic acid from the medium (Fig. 6). Uptake by

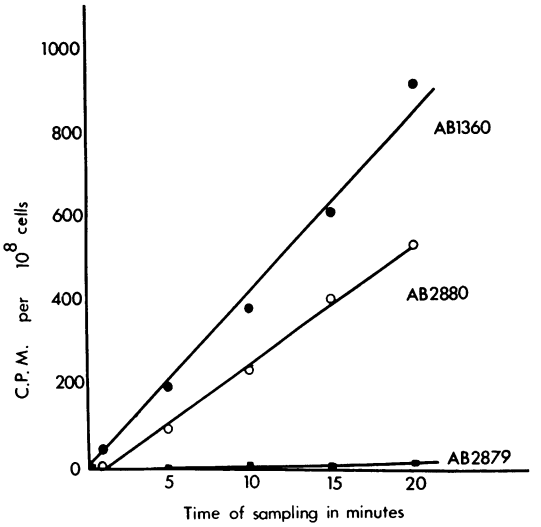

FIG. 5. *Uptake of C^{14}-shikimic acid by the parent strain AB1360 and by mutants AB2880 and AB2879.*

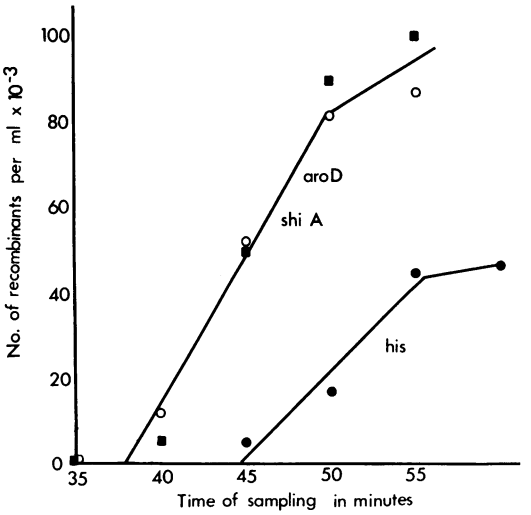

FIG. 4. *Kinetics of zygote formation when the Hfr AB259 was mated with the recipient AB2905 (a streptomycin-resistant derivative of AB2879).*

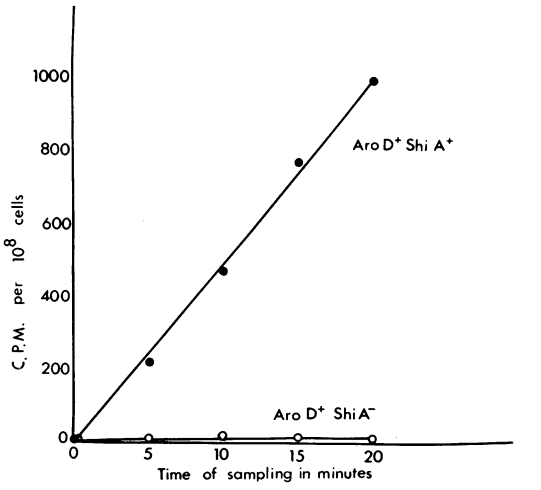

FIG. 6. *Uptake of C^{14}-shikimic acid by aroD$^+$ transductants of strain AB1360 (aroD$^+$ shiA$^+$) and strain AB2879 (aroD$^+$ shiA$^-$).*

the *aroD*$^+$ *shiA*$^+$ strain, on the other hand, resembled that already observed in AB1360 (*aroD*$^-$ *shiA*$^+$).

Common mechanism for the uptake of shikimic acid and dehydroshikimic acid. Strain AB1360 is blocked in the conversion of dehydroquinate to dehydroshikimate, but contains all the enzymes necessary for the conversion of dehydroshikimate to chorismate. Consequently, its aromatic requirements can be replaced by either shikimic acid or dehydroshikimic acid. Strain AB2879, on the other hand, is unable to grow on either shikimic acid or dehydroshikimic acid. In view of our conclusions regarding the function of the *shiA* gene, the simplest explanation of this result is that shikimic acid and dehydroshikimic acid enter the cell by a common mechanism.

DISCUSSION

The finding of mutant strains which have lost the ability to take up either shikimic acid or dehydroshikimic acid suggests a common transport mechanism or permease for these two intermediates in aromatic biosynthesis. Whether this permease is also concerned with the uptake of other compounds by the cell remains unknown, as we have failed to show any other alterations of cell phenotype associated with this particular mutation. We have not investigated the uptake of dehydroquinic acid, a third intermediate of aromatic biosynthesis, but we have examined these mutant strains for their ability to utilize a number of sugars and carboxylic acids as energy sources.

In addition to demonstrating the existence of a gene and, by inference, a permease concerned with the uptake of both dehydroshikimic acid and shikimic acid, these results demonstrate two of the difficulties that can be encountered in the interpretation of the results of simple genetic tests and nutritional tests when the nature of the mutations being studied is not properly understood. The first is that it is sometimes misleading to equate genotype with phenotype. In this case, it turns out that the genotypes *aroD*$^+$ *shiA*$^+$ and *aroD*$^+$ *shiA*$^-$ exhibit the same phenotype when examined by response to different growth factors but different phenotypes when examined for the ability to take up shikimic acid. The second is that in an apparently simple case, where a mutation has caused a growth requirement for a new amino acid (e.g., strain AB2880, showing a new requirement for tyrosine), it was shown that, contrary to expectation, the mutation did not affect one of the enzymes concerned in the specific reactions of biosynthesis of that amino acid.

ACKNOWLEDGMENTS

We thank Laurence Mason-Jones for excellent technical assistance, and Michael Edwards for the chorismic acid.

This investigation was supported by a grant from the Australian National Health and Medical Research Council, and by Public Health Service grant AM/4632 from the National Institute of Arthritis and Metabolic Diseases.

LITERATURE CITED

1. ADELBERG, E. A., AND S. N. BURNS. 1960. Genetic variation in the sex factor of *Escherichia coli*. J. Bacteriol. **79**:321–330.
2. ADELBERG, E. A., M. MANDEL, AND G. C. C. CHEN. 1965. Optimal conditions for mutagensis by N-methyl-N′-nitro-N-nitrosoguanidine in *Escherichia coli* K12. Biochem. Biophys. Res. Commun. **18**:788–795.
3. MONOD, J., S. COHEN-BAZIRE, AND M. COHN. 1951. Sur la biosynthèse de la β-galactosidase (lactase) chez *Escherichia coli*. La spécificité de l'induction. Biochim. Biophys. Acta **7**:585–599.
4. PITTARD, J., AND B. J. WALLACE. 1966. Distribution and function of genes concerned with aromatic biosynthesis in *Escherichia coli*. J. Bacteriol. **91**:1494–1508.