AROMATIC BIOSYNTHESIS

IV. Preferential Conversion, in Incompletely Blocked Mutants, of a Common Precursor of Several Metabolites

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Mutants of *Escherichia coli* have been isolated with blocks in a variety of reactions in aromatic biosynthesis. In addition to strains with single requirements for tyrosine, phenylalanine, tryptophan, and *p*-aminobenzoic acid, respectively, strains have been obtained with requirements for the first two, the first three, and all four of these compounds (Davis, 1950*a*). Moreover, most of the mutants with this quadruple requirement were found later to have an additional, relative requirement for a fifth compound, *p*-hydroxybenzoic acid (Davis, 1950*b*).

Certain intermediates in the biosynthesis of these five aromatic metabolites have been recognized. Some of these multiple aromatic auxotrophs can respond to shikimic acid (a 3,4,5-trihydroxycyclohexene-1-carboxylic acid; *cf.* figure 1) as a substitute for their requirement for two or more aromatic metabolites (Davis, 1950*a*); others accumulate this compound in the culture medium (Davis, 1951*a*); and still others accumulate a precursor of shikimic acid, provisionally designated as compound X (Davis, 1951*a*), which has been isolated and identified as 5-dehydroshikimic acid (Salamon and Davis, 1951*a*, *b*).

In the present paper the accumulations and nutritional responses of the 62 available multiple aromatic auxotrophs of several bacterial species will be summarized; and the double, triple, and quadruple auxotrophs of this series will be shown to be blocked in the same group of reactions as the quintuple auxotrophs. The differences in nutritional requirement will be shown to depend on (a) differences in the completeness of the genetic block, resulting in different limited rates of synthesis of a precursor, and (b) the preferential order in which this common precursor, when present in limited amounts, is converted to its various products.

On the basis of these observations a general scheme of aromatic biosynthesis, accounting for the behavior of all these strains, will be proposed. This scheme will include, in addition to the compounds mentioned, a previously unrecognized intermediate, compound W, and a previously unrecognized sixth metabolite derived from this series of intermediates. Finally, the nutritional requirements of the multiple aromatic auxotrophs will be shown to be at odds with the conclusion, based on inhibition analysis, that certain aromatic amino acids serve as precursors of others in *E. coli*.

METHODS

The cultural methods and the advantages of solid media in working with mutants have been described (Davis, 1951*a*). All experiments were performed

at 35 C in minimal medium A (Davis and Mingioli, 1950), supplemented as indicated; this medium contains glucose (0.2 per cent), citrate, and ammonium sulfate as sole carbon and nitrogen sources and has a pH of 7.0. Excretion of growth factors was detected by the technique of syntrophism, which has been illustrated elsewhere (Davis, 1950*a*). The chromatographic methods used for identification of the excreted compounds have been described (Davis, 1951*a*).

The mutants were isolated with the aid of the penicillin method (Davis, 1948, 1949; Lederberg and Zinder, 1948). Except as otherwise noted, they were derived from the W strain of *E. coli* (ATCC no. 9637). For mutants of other organisms we are grateful to Dr. Helen S. Vishniac, Dr. N. Zinder, Dr. J. Lederberg, Dr. P. Burkholder, Miss Elise Cahn, and the students in a summer course at the California Institute of Technology.

Shikimic acid was kindly furnished by Dr. K. Pfister of Merck and Company and by Dr. H. O. L. Fischer.

RESULTS

Growth requirements of the mutants. The available multiple aromatic auxotrophs of several species of Enterobacteriaceae and of *Bacillus subtilis* are listed in table 1. The requirements noted are those for growth at a rapid rate similar to that of wild type. The concentrations required for full growth on solid medium are L-tyrosine 20, L- or DL-phenylalanine 20 or 40, L-tryptophan 5, *p*-aminobenzoic acid 0.005, and *p*-hydroxybenzoic acid 0.01 μg per ml; these concentrations were used in compiling the data of table 1.

The strains are classified in table 1 in terms not only of their multiple requirements, but also of their ability to replace the required aromatic metabolites with various intermediates; these were tested as growth factors in concentrations of 10 to 200 μg per ml. It will be noted that strains can resemble each other in their utilization of these intermediates even though they differ from each other in their aromatic requirements.

Table 1 further shows that the mutants that resemble each other in their response to certain intermediates also resemble each other in their accumulation of others. These accumulations will be discussed below.

The mutants vary widely in stability, independently of the site of their block. Some (e.g., 83-1, 156-53, A170-143) are stable enough to provide excellent microbiological assays, which have been useful in the isolation of intermediates; others (e.g., 165A-50) are so unstable that a streak on minimal medium becomes overgrown with prototrophic reversions within a few days.

The sixth factor. At ph 7.5, but not at pH 7.0, the quintuple auxotrophs further require a sixth, unknown factor which is found in wild type filtrate and can be replaced by shikimic acid in low concentration. This sixth requirement also appears in media at pH 7.0 if, after mixing the agar with the other components, the media remain melted for a few hours at temperatures even as low as 45 C; this effect appears to depend on the formation of a toxic product from the agar. The nature of the sixth factor is under investigation.

Because of this sixth requirement it is necessary, in order to obtain rapid and

TABLE 1

Growth response and accumulation of multiple aromatic auxotrophs

Ty = tyrosine, Ph = phenylalanine, Tr = tryptophan, PA = *p*-aminobenzoic acid, PO = *p*-hydroxybenzoic acid, Sh = shikimic acid, DHS = 5-dehydroshikimic acid, W = unknown compound. Trace accumulations are indicated in parentheses; other accumulations are heavy (10–600 μg/ml). Mutants with numbers preceded by A, S, and BS were derived from *Aerobacter aerogenes*, *Salmonella typhimurium*, strain LT-22, and *Bacillus subtilis*, respectively; K, B, and X denote mutants of the K-12, B, and various recently isolated strains of *Escherichia coli*; the remaining mutants are from *E. coli*, strain W.

MUTANT NUMBER	AROMATIC REQUIREMENT FOR RAPID GROWTH	RESPONSE TO COMMON PRECURSORS	ACCUMULATION
83-20, 83-21, 83-24P1, S170-240, S170-302, S-170-313, S-170-389, S170-404	Double (Ty + Ph)	0	Sh,* (DHS, W)
83-4T, 83-22, 83-24, 159-8, BS-208	Triple (Ty + Ph + Tr)	0	Sh,* (DHS, W)
159-9, A170-9, A170-40, A170-44, S170-279, A188-13	Quadruple (Ty + Ph + Tr + PA)	0	Sh,* (DHS, W)
156-31, 159-2, 159-4, 159-7, 170-24, 170-108, 187-14	Quintuple (Ty + Ph + Tr + PA + PO)	0	Sh,* (DHS, W)
83-23, 83-25P1, A170-4, A170-138, S-38, X-1150, X-1426	Triple	Sh substitutes for requirement other than Ty, Ph	DHS, (W)
83-2, 83-25, K139-2, X-1104	Quadruple	Sh substitutes for requirement other than Ty, Ph	DHS, (W)
156-53, 165A-50, 165A-62, 170-18, 170-127	Quintuple	Sh substitutes for requirement other than Ty, Ph	DHS, (W)
K170-1	Double	Sh, DHS substitute for total req.	W
113–55	Quadruple	Sh, DHS substitute for total req.	W
83-1, 113-9, 121-143, 122-48, 159-1, 170-27, 171-38, B170-185	Quintuple	Sh, DHS substitute for total req.	W

TABLE 1—*Continued*

MUTANT NUMBER	AROMATIC REQUIREMENT FOR RAPID GROWTH	RETPONSE TO COMMON PRECURSORS	ACCUMULATION
83-26P1, BS-200	Triple	Sh, DHS substitute for total req.; W for part of req.	None known
83-26, X-1427	Quadruple	Sh, DHS substitute for total req.; W for part of req.	None known
83-3, 113-80, 159-5, 170-52, 171-33, A170-143	Quintuple	Sh, DHS substitute for total req.; W for part of req.	None known

* The mutants that accumulate shikimic acid also accumulate one or both of two unknown derivatives of this substance, compounds Z1 and Z2, whose positions in the biosynthetic scheme are uncertain.

reproducible growth of the quintuple aromatic auxotrophs on the quintuple supplement, to control the pH of the medium carefully, to sterilize the agar separately from the rest of the medium, and to pour the solid medium soon after mixing.

Genetic origin of multiple requirements. Each mutant described in table 1 is considered to owe its nutritional requirement to mutation of a single gene, resulting in loss of the activity (genetic block) of a single enzyme. Genetic support for this view is not obtainable as most of the mutants are derived from strains that have not shown genetic recombination. It is observed experimentally, however, as well as theoretically predictable, that the occurrence of more than one auxotrophic mutation in a single cell is rare in comparison with the occurrence of a single mutation. The repeated appearance of a given multiple aromatic requirement, therefore, offers, along with the obvious chemical relationship of the compounds involved, *prima facie* evidence for the origin of this requirement from a single mutation. In addition, on minimal medium most of our multiple aromatic auxotrophs have yielded spontaneous or ultraviolet-induced prototrophic reversions, apparently in a single step. With known multiple mutants, in contrast, prototrophic reversions, though observed (Witkin and Kennedy, 1951), appear to be very rare; we have been unable to obtain any from a wide variety of multiple mutants.

Since these multiple aromatic requirements appear to result from a single mutation, the strains with these requirements will be referred to as multiple auxotrophs or polyauxotrophs, in contradistinction to multiple mutants.

Biochemical origin of multiple requirements. A mutation would be polyauxotrophic in its effect if it inactivated an enzyme that synthesized a common precursor of several metabolites. Alternatively, the same effect could be pro-

duced if a genetic block in the synthesis of one metabolite resulted in the accumulation of a precursor that inhibited another synthesis, as was shown by Bonner (1946) for a Neurospora mutant, blocked in isoleucine synthesis, that required isoleucine plus valine. We found that in the corresponding mutant of *E. coli* the accumulated isoleucine precursor could be recognized easily by its growth factor activity for an isoleucine auxotroph blocked in an earlier reaction (unpublished observations). The aromatic polyauxotrophs, however, have given no indication of such a mechanism as they do not accumulate in the culture medium specific substitutes for any of the single aromatic amino acids. Furthermore, in all the polyauxotrophs the requirement for tryptophan can be satisfied by its earliest known specific precursor, anthranilic acid. Finally, the ability of most of the strains to satisfy their multiple requirement with a single precursor furnishes conclusive evidence for a block in a single early reaction.

Accumulation of precursors by aromatic polyauxotrophs. All the polyauxotrophs except those blocked in the earliest reaction (83-3 and similar strains) accumulate in their culture filtrates precursors that serve as growth factors for the 83-3 group (table 1). (The 83-3 group, of course, may also accumulate a precursor, but no mutant is available for its detection.) The accumulated substances were identified by paper chromatography combined with microbiological response, and on this basis the accumulations of some of the mutants listed in table 1 have already been reported (Davis, 1951*a*). The identification of the accumulated compounds has recently been completed by the isolation of shikimic acid (Shigeura *et al.*, 1951), 5-dehydroshikimic acid (Salamon and Davis, 1951*a*, *b*), and compound W (Weiss and Davis, 1952) from culture filtrates of strains 83-24, 83-2, and 83-1, respectively.

Some mutants accumulate as much as 600 mg of an intermediate per liter in aerated cultures; others accumulate less than 20 mg per L. The presence of an excess of the aromatic amino acids depresses the accumulation of all the precursors; various strains differ markedly in their sensitivity to this effect. Heavy accumulation of a given compound (e.g., shikimic acid) is usually accompanied by trace accumulation of its precursors (5-dehydroshikimic acid and compound W) as is noted in table 1.

Proposed scheme of aromatic synthesis. Compound W, 5-dehydroshikimic acid, and shikimic acid are shown in figure 1 as successive intermediates, for with this arrangement each mutant accumulates intermediates that precede its blocked reaction and utilizes those that follow this reaction.

In certain cases the utilization is limited; as was noted in table 1, shikimic acid can satisfy the requirement of 5-dehydroshikimic acid-accumulating strains for tryptophan, *p*-aminobenzoic acid, and *p*-hydroxybenzoic acid, but not for tyrosine and phenylalanine. This limitation is due to the accumulation of 5-dehydroshikimic acid, for this compound can be shown to interfere competitively with the further conversion of shikimic acid and particularly with its conversion to tyrosine and phenylalanine (Davis, 1952). Similarly, compound W cannot satisfy the tyrosine and phenylalanine requirements of 83-3 and related strains but can satisfy their other requirements. The details in this case have yet to be analyzed, but

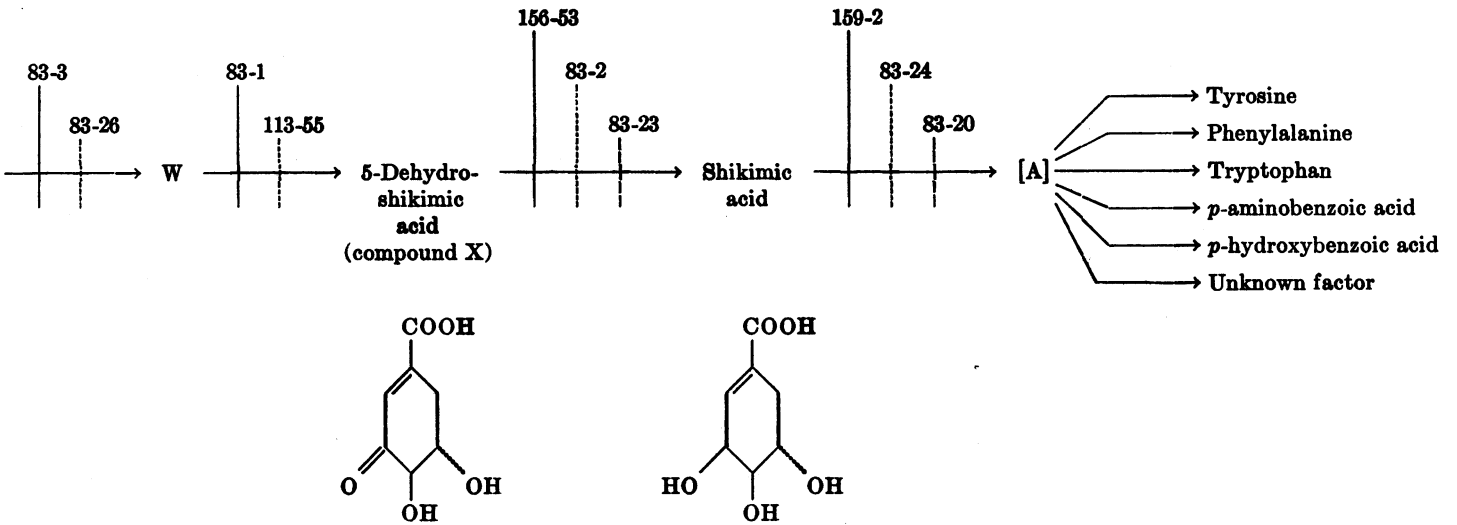

Figure 1. Scheme of aromatic synthesis

Solid and broken lines across arrows indicate complete and incomplete genetic blocks, respectively. The requirement for *p*-hydroxybenzoic acid is only relative; that for the sixth, unknown factor is hardly detectable unless the pH is raised to 7.5.

even the partial utilization justifies the assignment of a block before compound W.

The last known member of this series of aromatic precursors is shikimic acid. But some of the mutants that accumulate this compound have a quintuple and even, under appropriate conditions, a sextuple requirement. These strains are presumably blocked, like all the others described, in a single reaction, in this case one involving shikimic acid as substrate. Therefore, it has been necessary to postulate as the product of this reaction an even later common precursor of the six metabolites, compound A of figure 1.

The positions of the quintuple auxotrophs in this scheme present no special problem. The double, triple, and quadruple auxotrophs, however, require further discussion.

Postulation of incomplete blocks. In a scheme of aromatic synthesis proposed earlier (Davis, 1950*a*) the assumption was made that a double, triple, or quadruple requirement is caused by a genetic block before a specific common precursor of the two, three, or four required compounds, respectively; these strains, therefore, would be blocked in reactions later than the quintuple auxotrophs. This scheme, however, could not be reconciled with the fact that some triple auxotrophs do not accumulate as late intermediates as some quintuple auxotrophs do.

It was concluded, therefore, that the position of the block is indicated by the precursors accumulated and utilized, rather than the complexity of the nutritional requirement. In assigning genetic blocks on this basis (figure 1) the less exacting requirements have been accounted for by the assumptions that (a) the double, triple, and quadruple auxotrophs have incomplete blocks which result in the synthesis of limited amounts of a common precursor, and (b) this precursor, when present in limited supply, is preferentially utilized: it can satisfy the requirement for certain of its products but not the requirement for others. Further evidence for these assumptions is provided in the following sections.

Response to incomplete supplements. The supplements listed in table 1 are those required for rapid growth. The quadruple auxotrophs, however, with the exception of strain K139-2, grow, though slowly, on a triple supplement; they do not grow on a double supplement. The triple auxotrophs, in turn, respond in varying degrees to double or single supplements as is shown in table 2; most of them are accelerated by any single member or pair of the three amino acids, the most effective pair being tyrosine plus phenylalanine; and some strains (e.g., 83-21) are indeed difficult to classify as either triple or double auxotrophs. The double auxotrophs, in turn, are accelerated on minimal medium by either tyrosine or phenylalanine. Finally, the incomplete nature of the block is shown directly by the slow growth of the triple and double auxotrophs on minimal agar; and as would be expected from the postulated differences in the completeness of their genetic blocks, the double auxotrophs give rise to visible colonies earlier (two days) than the triples (three to five days) (table 2).

The relation of the triple requirement to p-aminobenzoic acid and carbon source. The arbitrariness of the classification of the various multiple auxotrophs accord-

ing to nutritional requirement is shown further by the effect of *p*-aminobenzoic acid on their growth. Careful observations on solid media occasionally showed slightly earlier appearance of visible growth of the so-called triple auxotrophs when *p*-aminobenzoic acid was added to the triple supplement. Further tests were performed, therefore, with a more sensitive turbidimetric method. Table 3 shows that all five triple auxotrophs tested were accelerated by the addition of *p*-aminobenzoic acid, indicating that these strains are blocked before a common precursor of *p*-aminobenzoic acid as well as of the three amino acids. In contrast, *p*-aminobenzoic acid did not accelerate either a double auxotroph, which pre-

TABLE 2

Effect of partial supplements and carbon source on growth of triple and double aromatic auxotrophs

The eight strains were streaked on a single plate of solid medium A, supplemented as indicated. Ty = L-tyrosine, 20 μg/ml; Ph = DL-phenylalanine, 40 μg/ml; Tr = L-tryptophan, 10 μg/ml. The glucose plates contained 0.2 per cent glucose; in the succinate plates glucose was replaced by 0.4 per cent sodium succinate. Readings of growth range from m (microscopic), ¼ (barely visible), and 1 (visible through the agar) to 4 (full growth). While the growth from day to day cannot be accurately measured by this technique, the readings on any one day were carefully compared.

CARBON SOURCE	SUPPLEMENT	MUTANT																								
		159-8			83-2P1			83-4P1			83-22			83-24			83-24P1			83-21			83-20		
		Growth at successive 24 hour intervals																								
Glucose	0	0	0	0	0	0	0	0	0	0	0	0	m	0	0	0	0	m	¼	0	¼	½	m	1	4
	Ty	0	m	m	0	0	0	0	m	m	0	m	m	0	m	m	m	½	1	0	¾	2	m	2	4
	Ph	0	m	m	0	0	0	0	m	m	0	m	m	0	m	m	m	¾	2	0	¾	3	m	2	4
	Ty, Ph	0	m	m	0	0	0	m	m	m	m	½	1	0	m	m	m	4	4	3	3	3	4	4	4
	Ty, Ph, Tr	2	3	4	3	4	4	3	3	4	3	3	4	2	3	4	3	4	4	4	4	4	4	4	4
Succinate	0	¼	¼	½	¼	¼	½	m	¼	½	¼	½	¾	½	½	1	1	3	4	½	¾	1	—	—	—
	Ty	¼	¼	½	¼	¼	½	m	¼	½	¼	½	1	½	1	2	3	4	4	½	2	3	—	—	—
	Ph	¼	¼	½	¼	¼	½	m	¼	½	¼	½	¾	½	½	1	2	4	4	½	2	3	—	—	—
	Ty, Ph	½	½	1	½	¾	1	½	¾	1	½	1	3	3	3	4	4	4	4	3	4	4	—	—	—
	Ty, Ph, Tr	2	3	4	4	4	4	2	2	2	2	3	4	3	3	4	4	4	4	4	4	4	—	—	—

sumably has a less complete block, or a phenylalanine auxotroph, which is not blocked before *p*-aminobenzoic acid.

Returning to table 2, we see that the genetic blocks in the triple auxotrophs appear even less complete when succinate is substituted for glucose in the medium; to a less marked extent the same effect has also been observed with lactate or acetate. The mechanism involved is obscure.

Further evidence on the sites of the blocks is provided by the effect of these mutations on the excretion of *p*-aminobenzoic acid. This excretion can be shown with the wild type by its heavy feeding of adjacent streaks of a *p*-aminobenzoic acid auxotroph within 24 hours (Davis, 1950*a*). Mutants with a single aromatic amino acid requirement behave identically with the wild type in this respect.

In contrast, the double auxotrophs show decreased excretion of *p*-aminobenzoic acid, while the triples show none at 24 hours and little or none at 48 hours. *p*-Aminobenzoic acid excretion is restored by an excess of shikimic acid (100 μg per ml) with the strains blocked before this compound but not with those blocked after it. These observations are all consistent with the scheme of figure 1.

TABLE 3

Acceleration of triple auxotrophs by addition of p-aminobenzoic acid

Fresh overnight cultures were washed with buffer and inoculated in 10 ml of medium A, supplemented as indicated. Growth was measured as in a preceding paper (Davis, 1951c). Triple supplement = L-tyrosine, 20 μg/ml; DL-phenylalanine, 40 μg/ml; L-tryptophan, 10 μg/ml.

AUXOTROPH	INOCULUM (ML)	PER CENT LIGHT TRANSMISSION			
		Supplement: triple		Supplement: same + PAB, 0.01 μg/ml	
		18 hr	24 hr	18 hr	24 hr
Single (phenylalanine):					
83-8	10^{-5}	50	49	49	48
	10^{-6}	82	49	78	48.5
Double:					
83-20	10^{-5}	93	47.5	92	48.5
	10^{-6}	100	75	100	62.5
Triple:					
159-8	10^{-5}	82.5	51	50.5	50
	10^{-6}	100	61	77	49.5
83-4P1	10^{-5}	97.5	48	58	48.5
	10^{-6}	100	97	92	50
83-22	10^{-5}	100	93.5	78	49
	10^{-6}	100	100	96	50
83-24	10^{-5}	100	96	54.5	48.5
	10^{-6}	100	100	84	50
83-23	10^{-5}	92	50	52	48.5
	10^{-6}	100	70.5	90	50

Utilization of 5-dehydroshikimic acid by a mutant incompletely blocked after it. Growth on minimal medium, which furnishes direct proof of an incomplete block in the double and triple auxotrophs, has not been observed with the quadruple auxotrophs. Another type of direct proof of an incomplete block, however, has been obtained for one of these strains (83-2). This mutant has a requirement intermediate between quadruple and triple and is blocked between 5-dehydroshikimic acid and shikimic acid. From this strain, as well as from a quintuple auxotroph (156-53) blocked, presumably completely, in the same reaction, sec-

ondary mutants were derived with an additional block located before 5-dehydroshikimic acid. These two double mutants, 83-2D2 and 156-53D2, respectively (figure 2), were nutritionally identical, each having a quintuple requirement and responding to shikimic acid as a sole growth factor (Davis, 1952).

If the requirement of strain 83-2 is less than quintuple because this strain can convert its accumulated 5-dehydroshikimic acid via shikimic acid to *p*-hydroxybenzoic acid and *p*-aminobenzoic acid, one might expect that the derived double mutant 83-2D2, though no longer accumulating 5-dehydroshikimic acid, could utilize added 5-dehydroshikimic acid to satisfy its *p*-hydroxybenzoic acid and *p*-aminobenzoic acid requirement, whereas in double mutant 156–53D2 the complete block between 5-dehydroshikimic acid and shikimic acid should prevent this substitution from being effective. This expectation was realized. Table 4 shows that in the presence of the three aromatic amino acids 5-dehydroshikimic acid supports slow growth of strain 83-2D2 but not of 156-53D2.

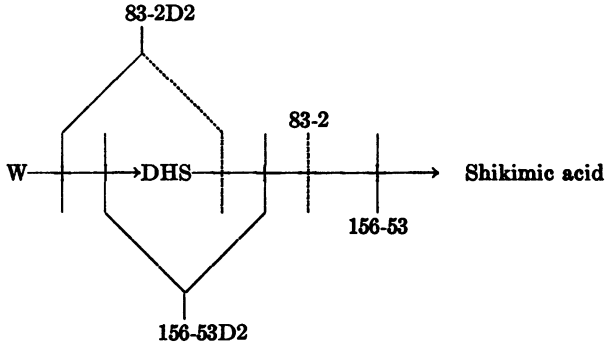

Figure 2. Sites of blocks in double mutants

Solid and dotted lines across arrows represent complete and incomplete blocks, respectively.

Table 4 also shows that the rate of utilization of 5-dehydroshikimic acid to satisfy the *p*-hydroxybenzoic acid and *p*-aminobenzoic acid requirement of strain 83-2D2 is essentially independent of 5-dehydroshikimic acid concentration over at least a thousandfold range. This result is somewhat surprising since the interference of 5-dehydroshikimic acid with shikimic acid utilization, also illustrated in table 4, has been shown to involve a constant competitive ratio (Davis, 1952). High concentrations of 5-dehydroshikimic acid, therefore, would be expected to prevent growth of strain 83-2D2, even in the presence of a triple supplement, unless antagonized by sufficient shikimic acid. As this shikimic acid presumably could arise in the present experiment only from 5-dehydroshikimic acid, the growth observed suggests that the effect of 5-dehydroshikimic acid concentration on shikimic acid production through the incomplete block in strain 83-2D2 may counterbalance its effect on the further conversion of the shikimic acid formed.

The order of preferential synthesis. Since the quadruple, triple, and double

auxotrophs clearly have, in the order given, increasing synthetic activity in their incompletely blocked reactions, it follows that limited formation of aromatic precursors gives rise to preferential synthesis of the derived metabolites in the order *p*-hydroxybenzoic acid, *p*-aminobenzoic acid, tryptophan, and finally phenylalanine and tyrosine. (Preferential synthesis refers here to preferential satisfaction of growth requirements, rather than to preferential synthesis in molar terms.)

This interpretation is supported by the isolation from many of the aromatic polyauxotrophs of secondary mutants with decreased growth requirements. For

TABLE 4

Utilization of 5-dehydroshikimic acid by a mutant blocked after it

Strain 83-2D2 has a presumably incomplete block (triple auxotrophic) after 5-dehydroshikimic acid, together with a complete block (quintuple auxotrophic) before it; strain 156-53D2 has complete blocks at both sites. Both strains were streaked on a single set of plates of minimal medium A supplemented as indicated below. Growth was estimated visually as in table 2. Triple supplement as in table 3.

SUPPLEMENT (μG/ML)			GROWTH			
	Shikimic acid	DHS	156-53D2		83-2D2	
			24 hr	48 hr	24 hr	48 hr
Triple	—	—	0	0	0	0
Triple + PAB 0.01 Triple + POB 0.01	—	—	2	3	3	4
Triple	0.1	—	3	4	3	4
Triple	0.1	1	3	4	3	4
Triple	0.1	10	0	m	1	3
Triple	—	0.1	0	0	½	2
Triple	—	1	0	0	½	2
Triple	—	10	0	0	½	2
Triple	—	100	0	0	¼	2
None	10	—	2	3	2	3
None	10	10	0	0	0	0

example, on a medium with a triple supplement, 5-dehydroshikimic acid-accumulating quadruple 83-25 spontaneously gave rise to a secondary mutant, 83-25P1 (table 1), which is indistinguishable in growth requirements and accumulation from some primary isolates with a triple requirement, such as 83-23. Similarly 83-24, a shikimic-accumulating triple auxotroph, gave rise in the presence of a double supplement to 83-24P1 (table 1), a shikimic-accumulating double auxotroph. These secondary strains are interpreted as partial reversions.[1] From vari-

[1] The metabolic interpretation of these reversions is not necessarily affected by the possibility that they may have arisen at a genetic locus different from the originally mutated one.

ous types of quintuple auxotrophs many other partial reversions have been obtained with the same quadruple, triple, and double requirements that have been observed in primary isolates. In contrast, attempts on appropriate media have failed to yield secondary mutants that had lost their requirements in a different order (e.g., tyrosine or phenylalanine before tryptophan).

This indirect evidence for preferential synthesis has been supplemented with direct evidence obtained with double mutant 156–53D2, blocked both before and after 5-dehydroshikimic acid. In this strain 5-dehydroshikimic acid acts as a competitive inhibitor of shikimic acid, and increasing ratios of 5-dehydroshikimic acid to shikimic acid could be shown to block successively the conversion of shikimic acid to tyrosine, phenylalanine, tryptophan, *p*-aminobenzoic acid, and *p*-hydroxybenzoic acid. Thus the effect of increasing degrees of chemical block supports the inference that the requirements of the various aromatic polyauxotrophs are due to various degrees of genetic block accompanied by preferential synthesis, for both chemical and genetic blocks produce an identical variety of growth requirements. The details of this work are presented elsewhere (Davis, 1952).

Significance of a relative p-hydroxybenzoic acid requirement; effect of aspartic acid, pH, and carbon source. Only one of the 25 available quintuple aromatic auxotrophs, W-accumulating strain 170-27, has an absolute *p*-hydroxybenzoic acid requirement. Most of the others require *p*-hydroxybenzoic acid only for rapid growth. The relative *p*-hydroxybenzoic acid requirement of these strains, however, is converted into an absolute one by L-aspartic acid (table 6), even in very low concentrations (2 to 10 μg per ml). The response of several such strains to various supplements is illustrated in table 1 of the accompanying paper (Davis, 1952).

It would be desirable to know whether the mutants whose *p*-hydroxybenzoic acid requirement is only relative resemble the double to quadruple auxotrophs in being incompletely blocked. Such a conclusion could be established readily for four of the quintuple auxotrophs since other parts of their requirement besides *p*-hydroxybenzoic acid are relative. Thus strains 156-31 and 165A-50 grow heavily on a triple supplement within three days; and 113-9 and 113-80 do so within two days, and even grow eventually on minimal medium. Aspartic acid, which delays growth of these strains in the absence of *p*-hydroxybenzoic acid, also delays their growth in the absence of any other component of the quintuple supplement.

In the remaining relative quintuple auxotrophs, however, which have an absolute quadruple requirement, it is not certain that the blocks are incomplete. For one thing, it would be expected that growth without added *p*-hydroxybenzoic acid, if dependent on the synthesis of this compound from a trace of a precursor formed through a slightly incomplete block, would be impeded more in a mutant with two such blocks than in a mutant with one; for a single block causes accumulation of an enormous concentration of the substrate of the blocked enzyme while an additional earlier block, sufficient to cause by itself a quintuple requirement, would at best allow this substrate to appear in a mere trickle. Yet the relative

p-hydroxybenzoic acid requirement observed in quintuple auxotroph 156-53, blocked after 5-dehydroshikimic acid, was not changed to a demonstrable extent in the strains derived from it, 156-53D2 and 156-53D31, which have an additional block before 5-dehydroshikimic acid. These results suggest that strain 156-53 may actually have a complete block in aromatic synthesis, despite its relative *p*-hydroxybenzoic acid requirement. This inference would naturally extend to the rest of the quintuple auxotrophs, except for the four noted previously which do not have a permanent quadruple requirement.

In this connection it should also be noted that the *p*-hydroxybenzoic acid requirement can be studied with quintuple auxotrophs only in the presence of a

TABLE 5

Replacement of p-hydroxybenzoic acid by p-aminobenzoic acid

The several mutants were streaked on the same plates of medium A without citrate, solidified with washed agar.* All plates contained the triple supplement listed in table 3, plus *p*-aminobenzoic acid and *p*-hydroxybenzoic acid as noted. Readings are scored as in table 2. Irr = irregular, large and small colonies.

PAB	MUTANT											
	83-1		159-4		170-27		83-1		159-4		170-27	
μg/ml	DL-Aspartic acid 0						DL-Aspartic acid 30 μg/ml					
	Growth at 24 and 48 hours											
0.01	m	2	m	2	0	0	0	0	0	0	0	0
0.1	¼	3	m	3	0	0	0	0	0	0	0	0
1.0	1	4	¼	3	0	0	0	0	0	0	0	0
20.0	2	4	2	4	0	Irr	0	Irr	0	Irr	0	±
0.01 + POB 0.01	3	4	3	4	3	4	2	4	2	4	2	4

* Traces of POB activity, as well as traces of other vitamins, could be demonstrated in unwashed compared with washed agar. It was found that these growth factors could be conveniently and effectively removed by washing the agar successively with 5 per cent clorox (5 minutes), water, 0.1 per cent sodium bisulfite, water, and alcohol.

quadruple supplement, that this supplement includes *p*-aminobenzoic acid, and that high concentrations of this compound (1 μg per ml or more) can accelerate the growth of the quintuple auxotrophs on a quadruple supplement, presumably through conversion of *p*-aminobenzoic acid to *p*-hydroxybenzoic acid.[2] This effect is shown in table 5. The possibility, therefore, can not be excluded that even the low concentration of *p*-aminobenzoic acid (0.01 μg per ml) present in the quadruple supplement might be the source of enough *p*-hydroxybenzoic acid to account for the slow growth of most quintuple auxotrophs on this supplement.

The main objection to this interpretation has been the absolute *p*-hydroxybenzoic acid requirement of strain 170-27; the existence of such a strain would

[2] *p*-Aminobenzoic acid, however, can hardly be on the main route of *p*-hydroxybenzoic acid synthesis since concentrations of *p*-aminobenzoic acid as high as 10 μg per ml produce less rapid growth than 0.01 μg per ml of *p*-hydroxybenzoic acid.

seem to imply a less complete block in strains with only a relative *p*-hydroxybenzoic acid requirement. Strain 170-27, however, may actually be no more

TABLE 6

Effect of various factors on requirements of quintuple aromatic auxotrophs

Strain 83-1, blocked between compound W and 5-dehydroshikimic acid, is representative of most of the quintuple aromatic auxotrophs; strain 170-27, though blocked in the same reaction, differs in several respects illustrated in this table.

The two strains were streaked on the same plates of medium A without glucose or citrate, supplemented as noted. Triple and quadruple supplements are those listed in table 3. Readings are scored as in table 2.

Qd = quadruple; Sh = shikimic acid.

CARBON SOURCE	pH	SUPPLEMENT	MUTANT							
			83-1		170-27		83-1		170-27	
%		μg/ml	DL-Aspartic acid 0				DL-Aspartic acid 100 μg/ml			
			Growth at 24 and 48 hours							
Glucose 0.2	7.0	Triple	0	0	0	0	0	0	0	0
		Qd	1	3	0	0	0	0	0	0
		Qd + POB 0.05	4	4	4	4	4	4	4	4
		Qd + Sh 0.05	4	4	4	4	4	4	4	4
Na succinate 0.4	7.0	Triple	¼	½	¼	½	¼	¼	¼	¼
		Qd	½	2	½	2	½	2	½	2
		Qd + POB 0.05	1	3	1	3	1	3	1	3
		Qd + Sh 0.05	3*	4	3*	4	3	4	3	4
Na succinate 0.4 + glucose 0.01	7.0	Triple	¼	¾	0	0	¼	½	0	0
		Qd	1	3	0	0	1	3	0	0
		Qd + POB 0.05	2	3	2	3	2	3	2	3
		Qd + Sh 0.05	3*	4	3*	4	3	4	3	4
Na acetate 0.2	7.0	Triple	¼	½	¼	¼	¼	½	¼	¼
		Qd	½	1	¼	¼	½	1	¼	¼
		Qd + POB 0.05	1	3	½	¾	1	3	½	¾
		Qd + Sh 0.05	2*	3	¾	¾	2	3	¾	¾
Na acetate 0.2 + glucose 0.01	7.0	Triple	¼	¼	¼	¼	¼	¼	¼	¼
		Qd	¾	3	¾	1	¾	3	¾	1
		Qd + POB 0.05	1	3	¾	1	1	3	¾	1
		Qd + Sh 0.05	2*	3	¾	1	2	3	¾	1
Glucose 0.2	6.0	Triple	0	¼	0	¼	0	¼	0	¼
		Qd	1	2	1	2	1	2	1	2
		Qd + POB 0.05	1	4	1	4	1	4	1	4
		Qd + Sh 0.05	1	4	1	4	1	4	1	4

* On media containing succinate or acetate the response to shikimic acid is better than that to POB. This effect is due to the fact that growth on these carbon sources, in contrast to that on glucose, is accompanied by an increase in pH; this change induces in these strains a sixth requirement which can be satisfied by shikimic acid.

completely blocked in aromatic synthesis than these others since its absolute *p*-hydroxybenzoic acid requirement appears only in certain media. Thus when glucose is replaced as carbon source by lactate, succinate, or acetate, strain 170-

27 exhibits as relative a *p*-hydroxybenzoic acid requirement as the other quintuple auxotrophs (table 6). And decrease of the pH of a glucose medium from 7.0 to 6.0 has an even more striking effect than replacement of glucose by succinate: not only does strain 170-27 lose its absolute *p*-hydroxybenzoic acid requirement, but the *p*-hydroxybenzoic acid requirement of all the strains virtually disappears (table 6).

It is noteworthy also that in a medium at pH 6.0 or in one containing no glucose, aspartic acid has no effect on the *p*-hydroxybenzoic acid requirement of any of the strains (table 6), even in concentrations of several hundred μg per ml; furthermore, the addition of even a little glucose (0.01 per cent) to a non-glucose-containing medium restores the absolute requirement of strain 170-27 without affecting the behavior of the other quintuple auxotrophs (table 6).

These facts suggest that strain 170-27 might differ from the other quintuple auxotrophs in having an additional mutation that exerts an effect on *p*-hydroxybenzoic acid metabolism similar to that exerted by aspartic acid. Further evidence for such an additional mutation is the fact that strain 170-27 differs from the other strains in utilizing acetate very poorly as carbon source, even in the presence of *p*-hydroxybenzoic acid (table 6).

In summarizing, it seems possible that most of the quintuple auxotrophs may have a complete block, despite their relative *p*-hydroxybenzoic acid requirement; but the alternative possibility of a slightly incomplete block has not been excluded.

DISCUSSION

Preferential synthesis following incomplete blocks. A general scheme of aromatic synthesis in various bacteria has been presented in which compound W (a metabolite of unknown structure), 5-dehydroshikimic acid, and shikimic acid function as successive intermediates in the synthesis of tyrosine, phenylalanine, tryptophan, *p*-aminobenzoic acid, *p*-hydroxybenzoic acid, and a sixth, unknown factor. This scheme is based on the accumulations and responses of a variety of mutants that require the five aromatic metabolites listed. In addition, mutants requiring the first two, three, and four of these compounds accumulate the same variety of precursors as the quintuple auxotrophs, and, therefore, are interpreted as being incompletely blocked at the same sites. Their growth requirements are explained by assuming that increasing formation of a common precursor through incomplete blocks results in preferential synthesis (in terms of the amounts needed to satisfy growth requirements) in the order *p*-hydroxybenzoic acid, *p*-aminobenzoic acid, tryptophan, and finally phenylalanine and tyrosine.

This assumption has been convincingly supported by several independent pieces of evidence. (a) The double and triple auxotrophs can grow, though slowly, on minimal medium, and hence must be incompletely blocked. (b) The "triple" auxotrophs are actually quadruple since their growth is accelerated slightly further by the addition of *p*-aminobenzoic acid. (c) Quintuple auxotrophs have given rise to partial reversions with nutritional requirements similar to those of the already described double to quadruple auxotrophs. (d) The slow utilization of a

precursor through a slightly incompletely blocked reaction has been directly demonstrated following the addition of a second, more complete block before this precursor. (e) Preferential synthesis has been directly demonstrated with a mutant whose growth is supported by shikimic acid and competitively inhibited by an analogue of this compound: different ratios of inhibitor to shikimic acid are required to interfere with the conversion of this metabolite to its various products, and the preferential order, from *p*-hydroxybenzoic acid to tyrosine, is the same as that inferred from the nutritional requirements of various aromatic polyauxotrophs.

It has been possible to account for most of the data presented here in terms of a fixed degree of "leakage" in each incompletely blocked mutant. The growth requirements of many of the strains described in this paper, however, can be affected by a variety of changes in the medium. Thus in 24 of the 25 available quintuple auxotrophs the *p*-hydroxybenzoic acid requirement is only relative; L-aspartic acid converts this relative requirement into an absolute one; replacement of glucose with succinate, lactate, or acetate causes the block to appear more incomplete in many double to quintuple strains; this replacement of glucose eliminates the aspartic acid effect on the *p*-hydroxybenzoic acid requirement of the quintuple auxotrophs (even though it does not entirely eliminate this requirement) and also eliminates the absoluteness of the *p*-hydroxybenzoic acid requirement in the one strain that has such an absolute requirement; decrease in pH largely eliminates the *p*-hydroxybenzoic acid requirement in all the quintuple auxotrophs; and increase in pH induces an additional sixth requirement in these strains. Similarly, other mutants of Neurospora or of bacteria are known to appear completely blocked under some circumstances, but to become partly or even wholly prototrophic on change of temperature, pH, or carbon source, or when vigorously aerated or when primed with a trace of the required factor (*cf.* Wagner and Haddox, 1951).

The intensifying effect of aspartic acid on the *p*-hydroxybenzoic acid requirement of quintuple auxotrophs parallels its synergism with the growth-inhibitory effect of *p*-hydroxybenzoic acid analogues on either these mutants or the wild type (Davis, 1951*b*). In both effects D-aspartic acid is inactive, and L-aspartic acid is active at extraordinarily low concentrations. Attempts to analyze the mode of action of aspartic acid have been inconclusive (*cf.* Davis, 1951*b*).

Equally obscure is the mechanism by which replacement of glucose with succinate or related compounds eliminates the ability of aspartic acid to induce an absolute *p*-hydroxybenzoic acid requirement. It is of interest that the same shifts of carbon source also have another striking effect; they allow tyrosine to be replaced by the corresponding α-keto acid as a growth factor for various mutants (Davis, 1951*a*). Neither of these actions of succinate and related compounds can be based on a simple pH effect since growth on these carbon sources causes the medium to become more alkaline—a pH change opposite to that which, on the ordinary medium, promotes utilization of ketotyrosine (Davis, 1951*a*) or growth of the quintuple auxotrophs without *p*-hydroxybenzoic acid.

The present experiments have shown that certain strains have slightly incom-

plete blocks even though they cannot grow on minimal medium. Such incomplete blocks without growth on minimal medium have also been demonstrated in isotope experiments with a quadruple aromatic auxotroph of Neurospora (Bonner, 1950) and with Neurospora mutants that respond to either tryptophan or niacin (Bonner *et al.*, 1952). In addition, obviously incomplete blocks, resulting in only a relative requirement, are frequently encountered but have not been extensively studied.

The order of preferential synthesis of the aromatic metabolites parallels their quantitative growth requirements: the requirement for *p*-hydroxybenzoic acid or *p*-aminobenzoic acid is about 1/1,000 that for tryptophan, which in turn is 1/4 that for tyrosine or phenylalanine. In addition to this consideration, however, other factors, such as varying affinity of different enzymes for a common substrate, may also be involved in preferential synthesis.

Preferential synthesis in incompletely blocked mutants may be widespread. This mechanism, for example, is suggested by Teas' (1950) observation of the synthesis of methionine but not threonine in a Neurospora mutant (strain 46003) that appears to be incompletely blocked in the formation of homoserine, a common precursor of these two metabolites. Preferential synthesis has been reported also to follow limitation in the supply of a catalyst, *p*-aminobenzoic acid, which participates (presumably after conversion to a coenzyme) in the synthesis of several metabolites. When the amount of *p*-aminobenzoic acid available is increasingly restricted as a result of sulfonamide inhibition (Kohn and Harris, 1943; Shive and Roberts, 1946; Winkler and de Haan, 1948) or of genetic block (Lampen *et al.*, 1949; Davis, 1951*c*), bacteria successively lose the ability to synthesize methionine, serine, purines, thymine, and other metabolites.

The general topic of competition of enzymes for a common substrate has been reviewed by Potter and Heidelberger (1950).

Absence of interconversion of amino acids. The data presented here have certain implications that conflict with the inferences of Beerstecher and Shive (1946, 1947) from studies based on inhibition analysis. These investigators concluded that in *E. coli* tryptophan acts as a precursor of phenylalanine, and in turn phenylalanine acts, as in the rat (Moss and Schoenheimer, 1940; Womack and Rose, 1946), as a precursor of tyrosine.

The latter conclusion has been criticized by Simmonds *et al.* (1947) since tyrosine fails to exert a sparing action on the quantitative requirement of a phenylalanine auxotroph. This observation, which we have confirmed, conflicts with the view that phenylalanine is a normal precursor of tyrosine.

This evidence, however, does not exclude the possibility of interconversion of these amino acids in the wild type via another path: reversal of the normal biosynthesis of phenylalanine to yield a precursor common to it and to tyrosine; for the genetic block in a phenylalanine auxotroph presumably would prevent both forward and reversed biosynthesis between the common precursor and phenylalanine. In the quintuple auxotrophs, in contrast, the blocks occur before the common precursor and consequently offer no obstruction to such interconversion by reversal of a normal path. Therefore, it is particularly significant that

the growth of these mutants is prevented by omitting any single aromatic amino acid from an otherwise adequate medium. This result excludes the possibility that any aromatic amino acid can be converted to another in these strains by either a normal or a reversed biosynthetic reaction.

To be sure, it is still possible that wild type *E. coli* might be able to convert one of these compounds to another, either by normal or by reversed biosynthesis; for the inability of a mutant to carry out this conversion might conceivably be due to a secondary effect of the mutation, such as inhibition of the conversion by an accumulated precursor. This possibility, however, seems remote since the absolute requirement for the three aromatic amino acids is found equally in mutants which are blocked in any one of at least four different reactions, and which consequently accumulate different precursors. It seems reasonable, therefore, to conclude that the normal path does not proceed from any one of these three amino acids to another.

Beerstecher and Shive (1946, 1947) further concluded that phenylpyruvic acid is not a precursor of phenylalanine. Simmonds *et al.* (1947), however, found that a phenylalanine auxotroph responded about equally well to either compound. We have confirmed this finding and have obtained similar results with a variety of aromatic polyauxotrophs (Davis, 1951*a*). It appears, therefore, that phenylpyruvic acid can serve as a precursor of phenylalanine.

We thus see several contradictions between the conclusions drawn from inhibition studies and those drawn from mutant studies. While both approaches contain pitfalls, these results suggest that inhibition antagonisms should be interpreted with particular caution. A further comparison of these two methodologies has been presented elsewhere (Maas and Davis, 1950).

SUMMARY

Mutants of *Escherichia coli*, *Aerobacter aerogenes*, *Salmonella typhimurium*, and *Bacillus subtilis* that require two, three, four, or five aromatic compounds have been shown to be blocked in the synthesis of precursors common to all five of the compounds. The multiplicity of the requirement depends on the completeness of the block; increasing inability to synthesize the common precursors leads to successive growth requirements in the following order: tyrosine, phenylalanine, tryptophan, *p*-aminobenzoic acid, and finally *p*-hydroxybenzoic acid.

The recognition of these incomplete blocks and of this preferential synthesis has allowed the growth requirements of the 62 available multiple aromatic auxotrophs to be reconciled with their accumulation and utilization of intermediates. A general scheme of aromatic biosynthesis, therefore, could be constructed, including the common precursors, compound W, 5-dehydroshikimic acid, and shikimic acid.

Most of the quintuple auxotrophs have only a relative *p*-hydroxybenzoic acid requirement, which becomes absolute in the presence of L-aspartic acid and disappears with decrease of pH to 6.0. Conversely, increase of pH to 7.5 induces a requirement for a sixth product of the common precursors. Some effects of change of carbon source on aromatic requirements are also described.

Since tyrosine, phenylalanine, and tryptophan cannot replace each other as growth factors for the quintuple auxotrophs, it is concluded that the bacterial species studied, in contrast to the rat, cannot utilize any one of these amino acids as a precursor of another.

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