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The Biosynthesis of Tyrosine from Labeled Glucose in *Escherichia coli*[*]

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ABSTRACT: The pathway of biosynthesis of tyrosine in *Escherichia coli* was investigated with labeled glucose. C-6 of glucose was incorporated into the ring of tyrosine only in C-2,6 and to the extent of 1.1 atoms. This result is identical with that observed earlier in the ring of shikimic acid, a precursor of tyrosine and several other aromatic metabolites. Similar correspondence between the rings of tyrosine and shikimic acid had already been observed with [1-^{14}C]glucose in yeast. Confirmation is therefore provided for the earlier conclusion, based on the enzymic reactions involved in the formation and function of prephenic acid, that in the conversion of shikimate to tyrosine the ring remains intact, C-1 to C-6 of shikimate corresponding to C-1 to C-6, respectively, of tyrosine. In view of the known origin of the rest of the ring of shikimic acid, these results are in conflict with the observation of other workers that in *Aerobacter aerogenes* [3,4-^{14}C]glucose gives rise to extensive labeling in three successive atoms, either C-2,3,4 or C-4,5,6, of tyrosine. Certain limitations of the degradation of labeled tyrosine are discussed, and a modification of the procedure is introduced.

Several lines of evidence have shown that shikimic acid is an intermediate in the biosynthesis of tyrosine, phenylalanine, and tryptophan as well as certain aromatic vitamins (Davis, 1951, 1955; Tatum *et al.*, 1954). On structural grounds it seemed reasonable to assume that in the formation of the benzene ring of these compounds the carbon ring of shikimate would remain intact.

This assumption was supported by the results of enzymic and other studies on the conversion of shikimate (I) to phenylalanine, tyrosine, and tryptophan (Scheme I). Shikimate 5-phosphate (II), formed from shikimate and adenosine triphosphate (Fewster, 1962), was shown to react with enolpyruvate phosphate to yield 3-enolpyruvylshikimate 5-phosphate (III) (Levin and Sprinson, 1964). Elimination of orthophosphate from III gave chorismic acid (IV) (Clark *et al.*, 1964), which was originally isolated from culture filtrates of a multiple mutant of *Aerobacter aerogenes* and shown to be the branch point intermediate for the formation of prephenate (V) and anthranilate (VI) (Rivera and Srinivasan, 1963; Gibson and Gibson, 1964). Hence, the carboxyl group on C-1 of prephenate is the original carboxyl of shikimic acid. This carboxyl group is lost during aromatization of prephenate (Katagiri and Sato, 1953; Davis, 1953), both to phenylpyruvate (VII) by prephenate dehydratase (Weiss *et al.*, 1954; Gilvarg, 1955) and to *p*-hydroxyphenylpyruvate (VIII) by prephenate dehydrogenase (Schwinck and Adams, 1959). The detailed knowledge now available of the reactions leading to the formation of the benzene rings of phenylalanine, tyrosine, and tryptophan from shikimate (I) indicates, on structural and mechanistic grounds, that the C–C bonds of the ring of shikimate remain unbroken throughout these conversions.

Since the pyruvic acid side chain and the carboxyl of prephenate (V) are attached to C-1 of shikimate these findings further suggested that carbon atoms 1–6 of the ring of shikimate become atoms 1–6, respectively,

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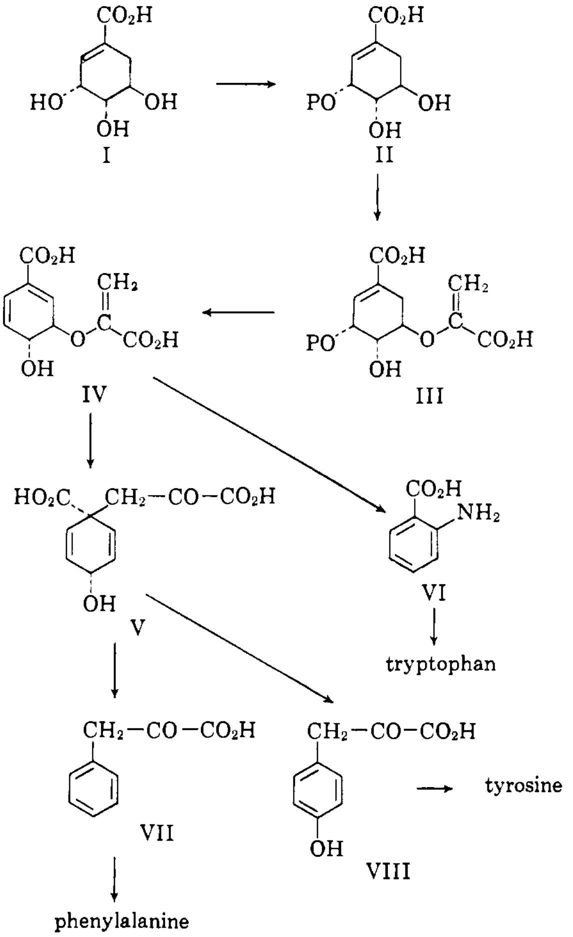


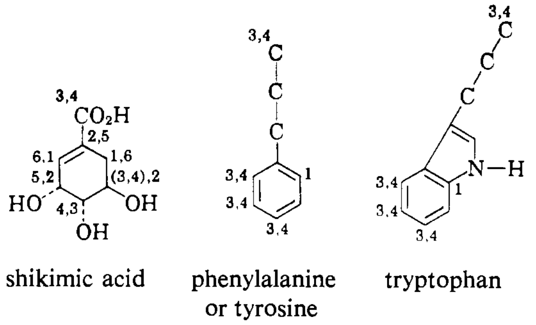
SCHEME I

of the benzene ring. The labeling of the latter ring from glucose should therefore be predictable from the results of studies on the incorporation of [^{14}C]glucose into shikimic acid in *Escherichia coli* (Srinivasan *et al.*, 1956).

However, it is difficult to reconcile this prediction with findings on the labeling of aromatic amino acids in *Aerobacter aerogenes* (Rafelson *et al.*, 1955; Rafelson, 1955; Ehrensvard, 1958). The results of the various tracer studies are summarized in Scheme II.

In the reported labeling of the ring of shikimic acid (Srinivasan *et al.*, 1956) the following features are noteworthy: the contribution of C-3,4 of glucose is limited to two consecutive positions; one of these (4 of shikimate) arises completely and the other (3 of shikimate) only partly from this source, and an adjacent atom (2 of shikimate) is partly derived from C-1 of glucose. In contrast, in the reported labeling of the benzene rings (Rafelson *et al.*, 1955), three consecutive positions are derived from C-3,4 of glucose, each is labeled to the extent of about 0.7 atom, and none is adjacent (*ortho*) to labeling from [1-^{14}C]glucose. It is clear that these reported isotope distributions are incompatible with aromatization of the intact ring of shikimic acid formed by the same path in the various organisms studied.

Since a difference in the aromatic pathway in these



SCHEME II: Incorporation of activity from labeled glucose into various positions in shikimic acid (Srinivasan *et al.*, 1956), tyrosine or phenylalanine (Rafelson *et al.*, 1955),[1] and tryptophan (Rafelson *et al.*, 1955; Rafelson, 1955). The numbers outside the rings represent carbon atoms of glucose reported to have been significantly incorporated.

closely related organisms seemed unlikely, the biosynthesis of tyrosine has been studied in the same species and under the same experimental conditions as were used for the earlier tracer work on shikimate. As C-1 and C-6 of glucose have a characteristic distribution in shikimate and the distribution of C-1 of glucose in tyrosine is already known (Gilvarg and Bloch, 1952), a determination of the distribution of C-6 of glucose in tyrosine should furnish critical evidence on the fate of the ring during conversion of shikimate to tyrosine. The results indicate that the ring does remain intact.

In this work an economy in labeled material was effected by using an *E. coli* mutant that excretes large amounts of tyrosine. Part of these results has been reported in preliminary form (Srinivasan *et al.*, 1954).

The pattern of incorporation of [3,4-^{14}C]glucose into anthranilate, the precursor of the benzene ring of tryptophan, is presented in the succeeding paper (Srinivasan, 1965), and is in complete accord with the results obtained with tyrosine.

Experimental Section

Accumulation and Isolation of Tyrosine. The organism used was *E. coli* mutant 83-5, a phenylalanine auxotroph that is blocked between prephenic acid and phenylpyruvic acid (Davis, 1953). This organism accumulates large amounts of tyrosine in the culture fluid when grown aerobically in the presence of a growth-limiting concentration of phenylalanine (Davis, 1950).

A fresh, washed culture (approximately 4 × 10^9 cells in 4 ml) was inoculated in 4 l. of minimal medium (Davis and Mingioli, 1950), supplemented with 32 mg of DL-phenylalanine and with 20 g of [6-^{14}C]D-glucose

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[1] The individual carbon atoms of C-2,6 (or C-3,5) of phenylalanine and tyrosine cannot be distinguished from each other. However, the benzene ring of tryptophan is not symmetrical, and so the incorporation of label into the individual carbon atoms can be determined. The assignment of activities shown in tyrosine and phenylalanine (Rafelson *et al.*, 1955) is based on analogy with the results obtained for tryptophan (Rafelson, 1955).

(autoclaved separately in 200 ml of water). After incubation at 37° with shaking for 48 hr the culture was acidified to pH 2, heated to 80° to drive off $^{14}CO_2$, and filtered through Celite. The filtrate was found, by bioassay with *E. coli* mutant 83-9, to contain 70 mg of L-tyrosine per liter. After bringing the filtrate to pH 1 the tyrosine was adsorbed on charcoal (Darco G-60), eluted with acetic acid–phenol (Partridge, 1949), isolated by isoelectric crystallization, and recrystallized to constant activity, $[\alpha]^{26}_{D}$ −10.3° (*c*, 5% 1 N HCl); recovery, 172 mg (58%).

Labeled Compounds. [6-^{14}C]D-Glucose was prepared by Dr. H. T. Shigeura as previously described (Srinivasan *et al.*, 1956). [4-^{14}C]Tyrosine was isolated from rat liver slices that had been incubated with [4-^{14}C]L-phenylalanine (Dische and Rittenberg, 1954).[2] The tissue was homogenized in ethanol and dried, and after thorough washing with 5% trichloroacetic acid the residue was hydrolyzed with 6 N HCl. The hydrolysate was freed of volatile acid, and tyrosine was isolated as described above.

Determination of Radioactivity. Samples were counted under standard conditions at "infinite" thickness in stainless steel or Teflon dishes of 1 cm^2 area. Unless otherwise stated the observed activities (corrected for background) were greater than background count and were determined with a probable counting error of 5%. The results are expressed as counts per minute divided by the fraction of carbon in the compound and multiplied by the number of carbon atoms per molecule; these values are proportional to the activity per mole. Where $BaCO_3$ was derived from more than 1 carbon atom of tyrosine the value was further multiplied by the number of carbon atoms represented. These relative molar activities are additive and permit the various degradative fragments to be directly compared to the labeled precursor.

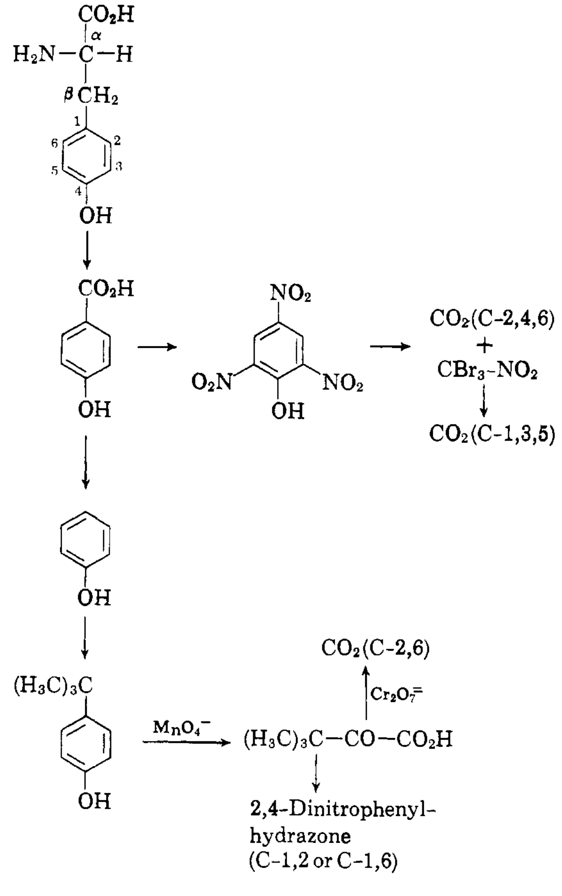
In the case of the trimethylpyruvic 2,4-dinitrophenylhydrazone, representing a mixture of C-1,2 and C-1,6 (see below), the value obtained was also multiplied by 2, yielding the activity of C-2,6 plus twice that of C-1. This figure is theoretically not additive but became so in the present experiments, in which the value of C-1 was 0.

Degradation of Tyrosine. The procedure was essentially that of Reio and Ehrensvärd (1953) except that bromopicrin was isolated in pure form prior to combustion (Badilley *et al.*, 1950). All operations were carried out exactly as described after several trial runs. Certain limitations of the procedure will be discussed under "Results."

p-Hydroxybenzoic acid and picric acid were purified to constant activity and reported melting point. *p*-Hydroxybenzoic acid (140 mg) was converted successively to phenol and *p*-*t*-butylphenol without purification of the products (Reio and Ehrensvärd, 1953). The *p*-*t*-butylphenol was oxidized with alkaline $KMnO_4$, and MnO_2 was removed by filtration. The filtrate was acidified and was treated with $K_2Cr_2O_7$ to obtain CO_2 (C-2,6), presumably resulting from the decarboxylation of trimethylpyruvic acid. The yield of $BaCO_3$ was 50 mg (25%). In some experiments (see "Results") the trimethylpyruvic acid (C-1,2 and C-1,6) was not oxidized but instead was isolated as a hydrazone by adding a 1% solution of 2,4-dinitrophenylhydrazine in 2 N HCl to the filtrate from the $KMnO_4$ oxidation. The yield of the crude hydrazone was 15 mg (4.5%). Following two crystallizations from ethyl acetate–petroleum ether the derivative was recovered in a yield of 50%; mp 170–172°; reported (Reio and Ehrensvärd, 1953) 171°.

Results

Evaluation and Modification of the Degradation Procedure. The reactions used for the degradation of tyrosine are shown in Scheme III. When this procedure was applied to tyrosine derived from [6-^{14}C]glucose certain difficulties were encountered. C-2,4,6 and C-2,6 were expected to have nearly equal activities, since C-4 of tyrosine was presumed to arise from C-4 of shikimic acid and hence (Scheme II) not significantly from C-6 of glucose. However, C-2,4,6 had an activity of 7800 cpm (Table I), while C-2,6, obtained as CO_2 from oxidation of the unpurified trimethylpyruvic acid fraction, had an activity of only 2000 cpm (not shown in Table I).



SCHEME III

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[2] We are greatly indebted to Dr. D. Rittenberg and Dr. M. R. Dische for making these tissue slices available to us.

TABLE I: Degradation of [4-^{14}C]Tyrosine and of Tyrosine Derived from [6-^{14}C]Glucose.[a]

Tyrosine or Degradation Products	Activities Derived from	
	Tyrosine from [6-^{14}C]-Glucose (cpm)[b]	[4-^{14}C]-Tyrosine (cpm)[b]
(1) Tyrosine	14,900	2200
(2) *p*-Hydroxybenzoic acid (C-1 to C-6 + β carbon)	14,800[c]	2230
(3) Picric acid (C-1 to C-6)	10,300[c]	2270
(4) C-1,3,5 (Br_3C-NO_2)[d]	0[c]	0
(5) C-2,4,6 (CO_2)[d]	7,800[c]	1990
(6) C-2,4,6 (calcd as (3) − (4))	10,300	2270
(7) 2,4-Dinitrophenylhydrazone of trimethylpyruvic acid[e]	7,200	660 ± 70[f]
(8) β Carbon (CO_2 from (2))	4,000	
(9) β Carbon (calcd as (2) − (3))	4,500	0

[a] The [6-^{14}C]glucose has an activity of 9300 cpm. [b] Relative molar activities, calculated as described under Experimental Section. [c] The average of two independent degradations. [d] From hypobromite oxidation of picric acid. [e] Calculated as described under Experimental Section and hence theoretically representing 2[(C-1) + 0.5(C-2,6)]. Since C-1 is inactive in these experiments, the values in the table should represent C-2,6. [f] Observed activity (corrected for background) from [4-C^{14}]tyrosine was only half of background count.

A modified procedure was therefore developed in which the trimethylpyruvate, instead of being oxidized without purification, was isolated as the 2,4-dinitrophenylhydrazone and counted. This fragment should be derived equally from C-1,2 and from C-1,6 of tyrosine. In the present experiment C-1,3,5 was inactive, and so the activity of the hydrazone (as calculated in Table I) should be that of C-2,6. On the basis of the assumption about C-4 noted above, this value should equal that of C-2,4,6. Satisfactory agreement was observed (Table I). However, both of these values appear to be appreciably low, as will be discussed below.

It is clear that the oxidation of the unpurified trimethylpyruvate fraction gave only about one-fourth the true value for the activity of C-2,6. It therefore appears that in this oxidation much of the CO_2 was contributed by sources other than the carboxyl group of trimethylpyruvic acid. Further support for this suggested explanation is furnished by the observation (see Experimental Section) that the yield of CO_2 from the trimethylpyruvate fraction was about three times the yield of hydrazone.

A more thorough check of the degradation procedure became possible through the availability of [4-^{14}C]tyrosine. With this compound, as with the tyrosine derived from [6-^{14}C]glucose, the sum of the activities of picric acid and of the CO_2 representing the β-carbon essentially equalled the activity of *p*-hydroxybenzoate (Table I). Furthermore, in the [4-^{14}C]tyrosine experiment the picric acid had the same activity as the *p*-hydroxybenzoic acid and the tyrosine. These portions of the degradation procedure are therefore reliable.

However, other portions of the degradation were less satisfactory. Thus the trimethylpyruvate hydrazone (Table I) obtained from [4-^{14}C]tyrosine, though expected to be inactive, was approximately 15% as active as the tyrosine. This finding suggests that the phenol was alkylated not only, as assumed, in the *para* position, but also in the *ortho* positions (C-3,5 of tyrosine). It is clear that even isolation of the trimethylpyruvic acid as a hydrazone did not ensure a reliable value for C-1,2,6 of tyrosine. In the degradation of the tyrosine derived from [6-^{14}C]glucose the activity of C-2,6 would be significantly underestimated.

To explain the activity in the hydrazone from [4-^{14}C]-tyrosine through alkylation of phenol in *ortho* positions (C-3,5 of tyrosine), the oxidation that followed would have to leave intact the tyrosine 3,4 or 4,5 bond (rather than the 2,3 or 5,6 bond). The method of calculation used, theoretically representing 2[(C-1) + 0.5(C-2,6)], would in this case become 2[(C-4) + 0.5(C-3,5)]. Hence the relative molar activity of C-4, calculated in this fashion, would be assigned twice its actual value. It follows that the value of 660 cpm for the hydrazone (Table I), which is 30% as high as the activity of the [4-^{14}C]tyrosine, must be divided by 2 in estimating the proportion of the trimethylpyruvic acid derived in the manner just described. (If any of the phenol alkylated in *ortho* positions were so oxidized as to lead to retention of the tyrosine 2,3 or 5,6 fragment rather than the 3,4 or 4,5 fragment, the activity of the resulting trimethylpyruvic acid would in the present experiments be the same as that yielded by the expected alkylation at C-1).

On the basis of the foregoing the activity of the trimethylpyruvic acid in the [6-^{14}C]glucose experiment might be corrected by a factor of 100/85 in calculating the activity of C-2,6. However, if the error is due to *ortho* alkylation it might be expected to vary from one experiment to another. More work would be required to establish definitely the source and constancy of the error in this portion of the degradation.

Difficulties were also encountered with the C-2,4,6 fragment obtained as CO_2 from hypobromite oxidation of picric acid. Since C-1,3,5 from both samples of tyrosine was inactive, the activity of C-2,4,6 should have been equal to that of the picric acid. However, C-2,4,6 was low by 24% in the [6-^{14}C]glucose experiment and by 13% in the [4-^{14}C]tyrosine experiment. Hence the CO_2 produced in the hypohalite reaction appears to be derived in part from sources other than C-2,4,6. (It has been recommended (Reio and Ehrensvärd, 1953; Badilley *et al.*, 1950) that a 10% correction be applied to this step on the basis of a CO_2 blank observed in control runs on the reaction mixture without picric acid. In our hands, however, such controls failed to yield CO_2.)

The Biosynthesis of Tyrosine from [6-^{14}C]Glucose. The uncorrected results of the degradation of tyrosine derived from [6-^{14}C]glucose (Table I) yielded a relative molar activity of 10,300 cpm (1.1 atoms of C-6 of glucose) for C-1 to C-6 of tyrosine and 7200 (0.8 atom) for C-2,6. These values, together with the inactivity of C-1,3,5, suggest an apparent incorporation of 0.3 atom of C-6 of glucose into C-4 of tyrosine. However, though the value for C-1 to C-6 (picric acid) can be considered reliable, the experimental determination of C-2,6 (trimethylpyruvic acid) was shown above to be low. This error could account for much and possibly all of the "missing" 0.3 atom. It can be concluded that the incorporation of C-6 of glucose into C-2,6 of tyrosine had a minimal value of 0.8 atom and a corrected value approaching 1.1. The results also showed that the incorporation of C-6 of glucose into the side chain of tyrosine amounted to about 0.5 atom and was located in the β-carbon atom.

Discussion

The Side Chain of Tyrosine. The observed incorporation of 0.5 atom of C-6 of glucose into the β carbon of the tyrosine side chain equals the previously reported incorporation of C-1 of glucose into the same atom (Gilvarg and Bloch, 1952). This equilibration of C-1 with C-6 of glucose is in accord with the origin of the side chain in enolpyruvate phosphate, a product of glycolysis (Scheme I).

The Rings of Tyrosine and Shikimic Acid. The observed incorporation of C-6 of glucose into the ring of tyrosine, which approaches 1.1 atoms in C-2,6, is similar to the previously reported incorporation of this atom into the ring of shikimic acid (Srinivasan *et al.*, 1956). C-1 of glucose shows similar agreement if one compares results obtained with tyrosine from the yeast *Saccharomyces* (Gilvarg and Bloch, 1952)[3] and with shikimic acid from *E. coli.* In both cases incorporation was essentially confined to C-2,6, and amounted to 0.5 atom in the tyrosine and 0.65 atom in the shikimic acid. It follows that *C-2,6 in both tyrosine and shikimic acid are derived largely from* carbons 1 and 6 of glucose.[4] In the succeeding paper (Srinivasan, 1965) the pattern of incorporation of C-3,4 of glucose into the ring of anthranilic acid, the precursor of the benzene ring of tryptophan, is shown to be identical with its incorporation into the ring of shikimic acid.

This correspondence between tyrosine and shikimic acid, together with the fact that the carboxyl group of shikimate has a different origin (Scheme II) from the β carbon of the tyrosine side chain, provides strong evidence that in the conversion of shikimate to tyrosine the carbon ring remains intact and the side chain displaces the carboxyl group on C-1. As indicated in the introduction, this conclusion is supported by the enzymic reactions involved in the formation of prephenic acid as well as in its conversion to phenylpyruvic acid and tyrosine. The present observations on the incorporation of label into tyrosine are incompatible with the report by Rafelson *et al.* (1955) that in *A. aerogenes* three consecutive atoms of the benzene ring of tyrosine (either C-2,3,4 or C-4,5,6) are derived largely from C-3,4 of glucose (Scheme II). The divergent results could be reconciled by assuming that the pathways of tyrosine biosynthesis are strikingly different, either for *A. aerogenes* compared with *E. coli* or for organisms grown under different conditions, or for the tyrosine excreted by a mutant (as in the present experiment) compared with that incorporated into protein. However, the explanation based on species difference seems remote. *A. aerogenes* not only is closely related to *E. coli* but also yields mutants that accumulate shikimic acid when grown on a complete aromatic supplement (Davis and Mingioli, 1953). Even in the more distantly related organism *Neurospora* the aromatic amino acids are synthesized *via* shikimic acid (Tatum *et al.*, 1954), and the isotope distribution from [1-^{14}C]glucose and [6-^{14}C]glucose to the shikimate ring is similar to that found in *E. coli* (Tatum and Gross, 1956). Furthermore, in plant lignins C-2,6 of the benzene ring of the phenylpropane residues is derived in a similar manner from [2,6-^{14}C]shikimate (Eberhardt and Schubert, 1956) and from C-1 and C-6 of glucose (Acerbo *et al.*, 1960). As to the possible influence of experimental conditions on isotope distribution, the only significant variable element in the known pathway from glucose to shikimate is exchange between the "upper" and the "lower" halves of the glucose chain preceding the formation of tetrose phosphate (Srinivasan *et al.*, 1956; Ljungdahl *et al.*, 1961), a precursor of ring carbons 4–6 (Srinivasan and Sprinson, 1959); within this variation the contribution of C-3,4 of glucose to the ring of shikimate would remain limited to two carbon atoms. It therefore appears that the reactions leading to the reported incorporation of C-3,4 of glucose into three consecutive carbon atoms of the benzene ring in *A. aerogenes* have yet to be explained.

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[3] A strain of *A. aerogenes* was also reported to incorporate carbon 1 of glucose into the ring of tyrosine and phenylalanine exclusively in C-2,6, but the extent of this incorporation was not given (Rafelson *et al.*, 1955).

[4] Similarly, in *Neurospora* carbon atoms 2 and 6 of protocatechuic acid, a metabolic product of 5-dehydroshikimic acid, are derived exclusively from carbons 1 and 6 of glucose (Tatum and Gross, 1956).

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The Biosynthesis of Anthranilate from [3,4-^{14}C]Glucose in *Escherichia coli*[*]

P. R. Srinivasan

ABSTRACT: *Escherichia coli* B-37 (a mutant blocked immediately after anthranilate) was grown in the presence of [3,4-^{14}C]glucose; the accumulated anthranilate was isolated and degraded to elucidate the pattern of labeling.

A comparison of this labeling with the pattern previously obtained in shikimate indicated that the carboxyl of shikimate becomes the carboxyl of anthranilate and the aromatization of the ring takes place without rearrangement. Furthermore, the method employed for the degradation revealed that the amination of an intermediate following 3-enolpyruvylshikimate 5-phosphate occurs on carbon 2 rather than on carbon 6 (based on shikimate numbering). In view of these new findings a scheme for the conversion of chorismate to anthranilate has been proposed.

Recent investigations with cell-free extracts have established that shikimate, shikimate 5-phosphate, and 3-enolpyruvylshikimate 5-phosphate are successive intermediates in the biosynthesis of anthranilate (Rivera and Srinivasan, 1963; Srinivasan and Rivera, 1963; Gibson *et al.*, 1962). The occurrence of yet another intermediate following 3-enolpyruvylshikimate 5-phosphate, *i.e.*, *Branch Point Compound*, has been proposed by us (Rivera and Srinivasan, 1963) as well as by Gibson and his colleagues (1962). This compound was recently isolated and characterized (Gibson and Gibson, 1964) and was also shown to be converted to anthranilate and prephenate (Gibson and Gibson, 1964; Clark *et al.*, 1964).

Earlier isotopic studies on the incorporation of [3,4-^{14}C]glucose into shikimate indicated that the carboxyl and carbon atoms 3 and 4 of the ring are labeled to the extent of 0.86, 0.59 and 0.9 atom, re-

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