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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-

* From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, N. Y. *Received July 9, 1965; revised September 15, 1965.* This investigation was supported by a research grant (GM 10384) from the National Institutes of Health.

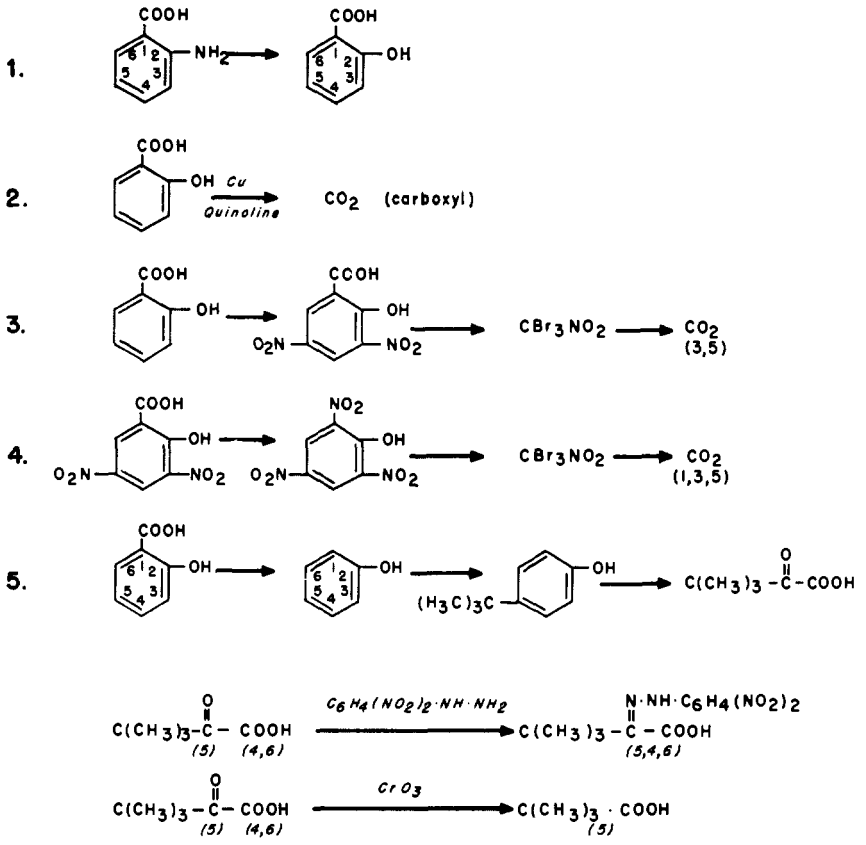


FIGURE 1: Reaction scheme for the degradation of anthranilic acid.

spectively (Srinivasan *et al.*, 1956). These isotopic studies, in conjunction with enzymatic investigations, suggest that the carboxyl of anthranilate should be derived from the carboxyl of shikimate; furthermore, only *two* carbon atoms of the ring of anthranilate (*i.e.*, 3 and 4) should be labeled from [3,4-^{14}C]glucose. However, these observations are at variance with the results reported by Rafelson (1955) on the incorporation of [3,4-^{14}C]glucose into tryptophan, namely, that three consecutive carbon atoms of the benzene ring are labeled. In the conversion of anthranilate to tryptophan the carboxyl is lost without any rearrangement of the benzene ring (Nyc *et al.*, 1949; Partridge *et al.*, 1952; Yanofsky, 1955). Thus the labeling pattern of the benzene ring of tryptophan should be identical with that found in the ring of anthranilate. In order to clarify this discrepancy and to gain further insight into the conversion of 3-enolpyruvylshikimate 5-phosphate to anthranilate, *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.

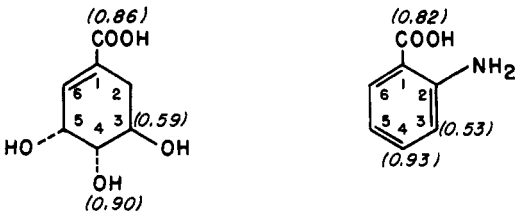
A comparison of this labeling with the pattern previously obtained in shikimate indicated that the carboxyl of shikimate becomes the carboxyl of anthranilate and the aromatization of the ring takes place without rearrangement. Furthermore, the method employed for the degradation revealed that the amination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

Experimental Procedure

Isolation of Anthranilic Acid. Escherichia coli mutant B-37 was grown with shaking on medium A (Davis and Mingioli, 1950) supplemented with 5 mg of tryptophan and 5 g of [3,4-^{14}C]glucose in a total volume of 1000 ml. Fifty hours later the culture medium was separated from the cells by centrifugation. The cells were washed once with 50 ml of H_2O and the washings were added to the original supernatant. The solution was then adjusted to pH 2 with 6 ml of 10 N H_2SO_4 and subjected to continuous ether extraction for 48 hr. The ether extract, after drying over anhydrous Na_2SO_4, was evaporated to dryness. The residue obtained was sublimed *in vacuo*. The sublimate (282 mg) was twice recrystallized from H_2O; yield: 202 mg; mp 143–144°.

Degradations. The [^{14}C]anthranilic acid was diluted with unlabeled anthranilic acid and degraded by the procedures outlined by Rafelson (1955) and Reio and Ehrensvärd (1952) with minor modifications. The degradation scheme is outlined in Figure 1.

Conversion of Anthranilic Acid to Salicylic Acid. To a solution of 508 mg of anthranilic acid in 6 ml of cold



SHIKIMIC ACID ANTHRANILIC ACID

FIGURE 2: Incorporation of carbon atoms 3 and 4 of glucose into shikimic acid and anthranilic acid.

H_2SO_4, 0.7 ml of cold $NaNO_2$ solution (0.4%) was added dropwise over a period of 10 min. Fifteen minutes later the diazotized solution was diluted with 12 ml of 4 N H_2SO_4 and was slowly added to a flask containing 12 ml of 4 N H_2SO_4 kept at 75° over a period of 15 min. The flask was shaken periodically and maintained at 75° for another 105 min. The crystals of salicylic acid were then collected by filtration and recrystallized from H_2O; yield: 374 mg; mp 155–156.5°.

Nitration of Salicylic Acid and Bromopicrin Cleavage of 3,5-Dinitrosalicylic Acid and Picric Acid. Salicylic acid (200 mg) was slowly added to a mixture at concentrated H_2SO_4 (0.55 ml) and fuming HNO_3 (1.42 ml; *d* 1.5) kept at 0°. After 10 min, 10 ml of H_2O was added and the solution was left at 2° for 3 hr. The dinitrosalicylic acid was filtered and recrystallized from 4 ml yf H_2O; yield: 155 mg; mp 176°. An additional yield of 18 mg could be obtained from the mother liquor.

Approximately 100 mg of 3,5-dinitrosalicylic acid was nitrated further by the method described by Reio and Ehrensvärd (1952); yield of picric acid: 35 mg; mp 121°.

The bromopicrin cleavage of the two nitro compounds was carried out on 20 mg of the materials by the method of Reio and Ehrensvärd (1952).

Conversion of Salicylic Acid to Phenol and Its Further Degradation to Trimethylacetic Acid. These conversions were carried out on 355 mg of salicylic acid by the method outlined by Reio and Ehrensvärd (1952) for the degradation of *p*-hydroxybenzoic acid. The *p*-*t*-butylphenol was oxidized to trimethylpyruvic acid without further purification. An aliquot of the trimethylpyruvic acid solution was withdrawn and treated with 1% 2,4-dinitrophenylhydrazine in 2 N HCl. The precipitated, 2,4-dinitrophenylhydrazone of trimethylpyruvic acid was collected and recrystallized from ethyl acetate–petroleum ether (mp 170–173°). The remainder of the original trimethylpyruvic acid solution was oxidized to trimethylacetic acid with chromic acid; the trimethylacetic acid formed was steam distilled and isolated as the sodium salt.

Preparation of [3,4-^{14}C]Glucose and Radioactivity Determinations. [3,4-^{14}C]Glucose was prepared by subcutaneous injection of [^{14}C]$NaHCO_3$ into fasted rats which had received glucose by stomach tube just prior to the injection (Arnstein and Bentley, 1953). After 2 hr the rats were sacrificed and the livers were removed and saponified with KOH to isolate glycogen. Glucose was then obtained by acid hydrolysis of the purified glycogen.

The radioactivity of the various samples was determined in a gas flow counter under standard conditions at infinite thickness in Teflon planchets 1 cm^2. The samples were counted long enough to give a probable error of 5%. Unless otherwise indicated, the counts per minute of the various samples were at least twice the background. The results are expressed as counts per minute under standard conditions (Srinivasan *et al.*, 1956). Wherever the $BaCO_3$ was derived from more than 1 carbon atom of anthranilic acid, the activity was further multiplied by the number of carbon atoms the $BaCO_3$ represented. In this form the data are additive and are directly comparable to the labeled precursor added to the medium.

Results and Discussion

The results of the degradation are presented in Table I. The fraction of [3,4-^{14}C]glucose incorporated into anthranilic acid, *i.e.*, 2.32 carbon atoms, is in agreement with that found previously in shikimate, *i.e.*, 2.35 carbon atoms. The activity of the carboxyl of anthra-

TABLE I: Degradation of Anthranilic Acid Derived from [3,4-^{14}C]Glucose.

Compound or Atom	Molar Activity (cpm)	Atoms of Glucose Incorporated
(1) Glucose	33,250[a]	
(2) Anthranilic acid	77,080	2.32
(3) Salicylic acid	77,000	
(4) 3,5-Dinitrosalicylic acid	77,180	
(5) Picric acid	49,000	
(6) C-3,5 (Bromopicrin)[b]	17,750	0.53
(7) C-1,3,5 (Bromopicrin)[c]	17,750	0.53
(8) 2,4-Dinitrophenylhydrazone of trimethylpyruvic acid[d]	31,000	0.93
(9) C-5 (trimethylacetic acid)	2,580	0.08
(10) Carboxyl (CO_2 from (3))	27,000	0.81
(11) Carboxyl (calcd as (4) − (5))	28,180	0.85

[a] This value represents the individual molar activity of carbon 3 and of carbon 4 of glucose, the observed molar activity being 66,500. [b] From hypobromite oxidation of 3,5-dinitrosalicylic acid. [c] From hypobromite oxidation of picric acid. [d] This fragment should be derived equally from C-5,4 and C-5,6 of anthranilic acid. A correction of 15% was applied to take into account the dilution by other carbon atoms (Sprecher *et al.*, 1965).

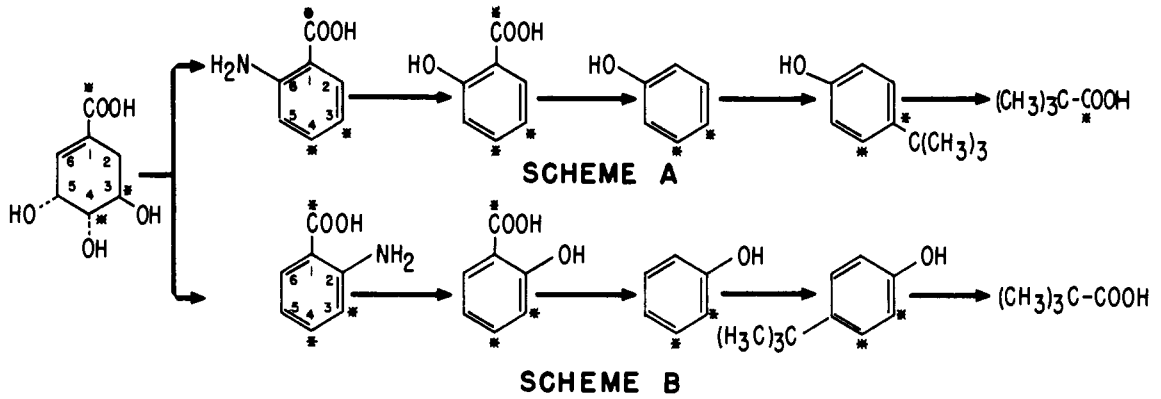


FIGURE 3: Degradation schemes illustrating the position of amination of shikimic acid.

nilic acid can be estimated by two methods: by direct decarboxylation of salicylic acid or by calculating the difference in molar activities between picric acid and dinitrosalicylic acid. The experimentally determined value closely corresponds to the calculated value (lines 10 and 11 in Table I). Since the activity of C-5 (line 9, Table I) is negligible it can be assumed that all the activity observed in C-3,5 resides in C-3. Moreover, the molar activities of C-1,3,5 and C-3,5 are identical, indicating that carbon 1 is essentially inactive. Since C-5 is inactive, the activity found in the 2,4-dinitrophenylhydrazone or trimethylpyruvic acid should represent either carbon 4 or 6. C-6 of shikimate is derived from C-1 and C-6 of glucose, and C-4 of shikimate arises from C-4 of glucose (Srinivasan *et al.*, 1956). It is therefore reasonable to assign the activity found in the trimethylpyruvic acid fragment to carbon 4 of anthranilic acid.

The small amount of radioactivity found in trimethylacetic acid could be attributed to a small but significant amount of *ortho* alkylation of phenol by *t*-butyl alcohol. A similar explanation can also be offered for the lowered molar activity observed in the trimethylpyruvic acid fragment isolated as the 2,4-dinitrophenylhydrazone (Sprecher *et al.*, 1965).

Figure 2 shows the fraction of glucose incorporated into anthranilate, and this is compared to the shikimate labeling reported previously. The pattern and the extent of incorporation in anthranilate parallels closely that found earlier in shikimate. In anthranilate only two carbon atoms of the ring, *i.e.*, 3 and 4, are labeled. These results also support our earlier observations on the biosynthesis of tyrosine from [6-^{14}C]glucose (Sprecher *et al.*, 1965). Although the present findings are different from those observed by Rafelson (1955), they are consistent with the enzymatic conversion of shikimate to anthranilate and also to prephenate, a precursor of tyrosine and phenylalanine.

The method employed for the degradation of anthranilate also permits us to unequivocally identify the position at which amination of shikimate occurs. This is illustrated in Figure 3. Since the carboxyl of anthranilate is derived from the carboxyl of shikimate, amination by glutamine can occur either at carbon 2 or 6 Either one of these reactions could yield anthranilate.

Alkylation of phenol by *t*-butyl alcohol occurs predominantly *para* to the hydroxyl group (Reio and Ehrensvärd, 1952). In Scheme A it is assumed that the amination occurs at carbon 6, in which case the trimethylacetic acid formed must possess all the activity found in carbon 3 of shikimate, *i.e.*, 0.59 of an atom of [3,4-^{14}C]glucose. In Scheme B, the amination was presumed to take place at carbon 2; such a situation would yield inactive trimethylacetic acid from the degradation of anthranilate grown on [3,4-^{14}C]glucose. In the present experiments the trimethylacetic acid was found to have negligible radioactivity. One must therefore conclude that the amination occurs at carbon 2 of shikimate.

Recently we have shown that the enzyme complex capable of forming anthranilate from 3-enolpyruvylshikimate 5-phosphate and L-glutamine can be further fractionated to yield fractions I and II, both of which were required to synthesize anthranilate from the above substrates. Inorganic phosphate was rapidly released from 3-enolpyruvylshikimate 5-phosphate in the presence of fraction I, and this nonphosphorylated intermediate was converted to anthranilate in the presence of fraction II and glutamine (Rivera and Srinivasan, 1963).

The nonphosphorylated compound is most probably chorismate,[1] a new intermediate isolated by Gibson and Gibson (1964). This is supported by the finding that chorismate is rapidly converted to anthranilate in the presence of enzyme fraction II (Table II). Moreover, fraction I from *E. coli* B-37 has been utilized by Clark *et al.* (1964) for the purification of chorismate synthetase, the enzyme which forms chorismate from 3-enolpyruvylshikimate 5-phosphate. Chorismate has also been shown to be an intermediate after 3-enolpyruvylshikimate 5-phosphate in the biosynthesis of both anthranilate and prephenate (Gibson and Gibson,

[1] The nonphosphorylated compound formed by fraction I could still be a later intermediate after chorismate, but this is rather unlikely.

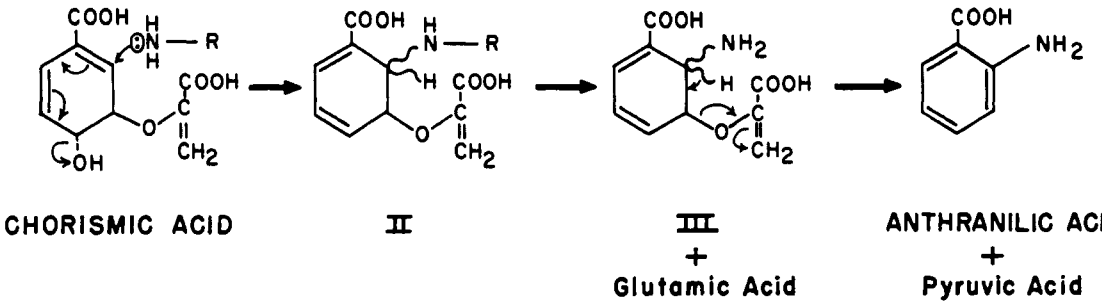


FIGURE 4: Postulated scheme for the conversion of chorismic acid to anthranilic acid (R–NH_2 = glutamine).

TABLE II: Synthesis of Anthranilate from Either 3-Enolpyruvylshikimate 5-Phosphate or Chorismate with Fractions I and II of *E. coli* B-37.[a]

Substrate	Enzyme Fraction[b] (2 mg of protein)	Anthranilate Formed (μmole)
3-Enolpyruvyl-shikimate 5-phosphate (0.5 μmole)	Fraction I	0
	Fraction II	0
	Fraction I + II	0.26
Chorismate (0.25 μmole)	(a) Fraction I	0
	Fraction II	0.24
	Fraction I + II	0.17
	(b) Fraction II	0.24
	(c) Fraction II	0

[a] The reaction mixture contained 2.5 μmoles of L-glutamine, 5 μmoles of $MgCl_2$, 5 μmoles of reduced glutathione, 50 μmoles of Tris buffer, pH 8.2, 0.5 μmole of NAD, 150 μmoles of ethanol, 0.15 mg of alcohol dehydrogenase, either 3-enolpyruvylshikimate 5-phosphate or chorismate, and enzyme fraction in a total volume of 1 ml. The mixture was incubated for 30 min at 37°. In experiment b the nicotinamide–adenine dinucleotide (NADH) regenerating system was omitted and in experiment c both NADH-regenerating system and $MgCl_2$ were omitted. [b] Details regarding the preparation of the enzyme fractions were described in an earlier publication (Rivera and Srinivasan, 1963).

1964; Clark *et al.*, 1964). As shown by the results presented in Table II, the conversion of chorismate to anthranilate requires only Mg^{2+}, indicating that the regenerating system for NADH is required in the prior conversion of 3-enolpyruvylshikimate 5-phosphate to chorismate.

The experimental evidence presented in this communication clearly establishes that in the formation of anthranilate amination occurs at carbon 2 of shikimate. On this basis the following tentative scheme for the conversion of chorismate to anthranilate is proposed (Figure 4). A nucleophilic attack by the amide N of glutamine at carbon 2 of chorismate will result in the formation of compound II, which is then converted to 2,3-dihydro-3-enolpyruvylanthranilate. Elimination of the enolpyruvyl group results in the production of anthranilate.

We have examined 2,3-dihydro-3-hydroxyanthranilate as a possible precursor to anthranilate in cell-free extracts of *E. coli* B-37 with negative results. This would imply that the true intermediate still bears the enolpyruvyl side chain before aromatization. Such a hypothesis is supported by the recent suggestive observation of Lingens *et al.* (1963) that a mutant of *Saccharomyces cerevisiae* accumulates a product similar to compound II. Alternatively compound III could be synthesized directly from chorismate without the formation of compound II as an intermediate.

Recently DeMoss (1965) has proposed that a single enzyme "anthranilate synthetase" catalyzes the conversion of chorismate to anthranilate in *N. crassa*. Compounds II and III might then be enzyme-bound intermediates. Whether a similar pathway also exists in *E. coli* and in yeast requires further exploration.

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CORRECTION

In the paper by Bruce Mackler, Howard C. Douglas, Susan Will, Donald C. Hawthorne, and Henry R. Mahler, in Volume 4, No. 10, October 1965, the following corrections should be made: p. 2016, right column, line 13 should read "plasmic ($P\rho^-$) and segregational (or genic) (($p \times \rho^+/\rho^-$); p. 2019, right column, line 13 should read "mutant ($P\rho^-$) as compared to its complete absence in an"; and p. 2020, left column, line 2 should read "sufficiency, *i.e.*, it is of the genotype $p_{12}\rho^-$. Conversely. . ."