The Role of 3-Enolpyruvylshikimate 5-Phosphate in the Biosynthesis of Anthranilate*

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Received April 3, 1963

The role of 3-enolpyruvylshikimate 5-phosphate in anthranilate biosynthesis has been studied in partially purified enzyme fractions of *E. coli* B-37. Fractionation of the crude extract of *E. coli* B-37 resulted in the separation of the anthranilate-forming enzyme system into two fractions (*i.e.*, fraction I and fraction II). Both enzyme fractions were required for the synthesis of anthranilate although in the presence of fraction I shikimate 5-phosphate disappeared rapidly. With the aid of these partially purified fractions I and II the following facts were established: (a) Shikimate 5-phosphate could no longer serve as a carbon source for the synthesis of anthranilate unless phosphoenolpyruvate were also included in the reaction mixture. Shikimate 5-phosphate and phosphoenolpyruvate could be replaced by their enzymatic condensation product 3-enolpyruvylshikimate 5-phosphate. (b) Fraction I contained the enzyme 3-enolpyruvylshikimate 5-phosphate synthetase required for the condensation of shikimate 5-phosphate and phosphoenolpyruvate. (c) In the presence of enzyme fraction I a new intermediate was formed from 3-enolpyruvylshikimate 5-phosphate again convertible to anthranilate, in the presence of L-glutamine, by appropriate extracts. (d) The synthesis of anthranilate from 3-enolpyruvylshikimate 5-phosphate and L-glutamine required Mg^{++}, NAD, and a NADH-regenerating system. The role of 3-enolpyruvylshikimate 5-phosphate in anthranilate biosynthesis was further confirmed by using extracts of mutants blocked immediately before and after this compound. In crude extracts of *E. coli* B-37 glutamine was serving the dual role of an amino donor and a source of phosphoenolpyruvate.

In the preceding paper (Srinivasan and Rivera, 1963) extracts of *Escherichia coli* mutant B-37 were shown to form anthranilate (an intermediate in the biosynthesis of tryptophan) from shikimate 5-phosphate in the presence of L-glutamine.[1] Shikimate could also serve as a carbon source provided ATP was added. These results were in complete agreement with the existence of quintuple auxotrophs which accumulated in their medium either shikimate or shikimate-5-P and required the supplementation of phenylalanine, tyrosine, tryptophan, *p*-aminobenzoate, and *p*-hydroxybenzoate for growth (Davis and Mingioli, 1953). Other quintuple auxotrophs with the same requirements accumulated 3-enolpyruvylshikimate in their medium (Davis and Mingioli, 1953). The latter compound, however, was found not to substitute for shikimate-5-P as a carbon donor in the biosynthesis of anthranilate.

Recent investigations (Levin and Sprinson, 1960) on the enzymatic synthesis of 3-enolpyruvylshikimate indicated that it arose from a dephosphorylation of 3-enolpyruvylshikimate-5-P (ESP), the latter being derived by a condensation of shikimate-5-P and phosphoenolpyruvate (PEP). These new observations suggested the possibility that ESP and not its dephosphorylated derivative 3-enolpyruvylshikimate is the actual intermediate in aromatic biosynthesis.

The results to be reported here support the conclusion that ESP is an intermediate in the biosynthesis of anthranilate. The partial purification of the anthranilate-forming enzyme system facilitated such a study. In crude extracts glutamine served the dual role of an amino donor and a source for PEP. Evidence will also be presented for the existence of another new intermediate after ESP in anthranilate biosynthesis. A part of these results has appeared in preliminary form (Rivera and Srinivasan, 1962).

EXPERIMENTAL PROCEDURE

Materials.—Shikimate-5-P was isolated from the culture medium of *Aerobacter aerogenes* A170-40 as the barium salt (Weiss and Mingioli, 1956). 3-Enolpyruvylshikimate-5-P was prepared by the enzymatic condensation of shikimate-5-P and phosphoenolpyruvate by the method described by J. Levin (1962).

Preparation of Bacterial Extracts.—*E. coli* B-37 was grown for 18 hours with aeration in medium A (Davis and Mingioli, 1950) supplemented with 5 μg of tryptophan per ml. *A. aerogenes* mutants A170-40 and A170-44 were grown on medium A supplemented with 20 μg each of phenylalanine and tyrosine, 5 μg of tryptophan, 0.02 μg each of *p*-aminobenzoic acid and *p*-hydroxybenzoic acid per ml. *Salmonella typhimurium* mutants try A-2, try A-6 and try A-8 were grown on medium A supplemented with 5 μg of tryptophan per ml. Mutant A-2 also required arginine (20 μg/ml) for growth.

Extracts of the cells were prepared by disrupting 5 g of cells in 20 ml of 0.033 M Tris buffer, pH 7.4, containing reduced glutathione (10^{-4} M) and Versene (10^{-4} M), by sonic oscillation for 30 minutes in a 9-kc Raytheon Sonic Oscillator. Further operations were carried out as described in the preceding paper (Srinivasan and Rivera, 1963). In the case of *A. aerogenes* mutants 10 g of cells was used for every 20 ml of buffer. The extracts prepared in the manner described above consistently gave high enzyme activity from different batches of cells.

* This investigation was supported by a grant (GM-10384-01) from the National Institutes of Health, U. S. Public Health Service.

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[1] The following abbreviations are used: Shikimate-5-P, shikimate 5-phosphate; PEP, phosphoenolpyruvate; ESP or 3-enolpyruvylshikimate-5-P, 3-enolpyruvylshikimate 5-phosphate; DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate.

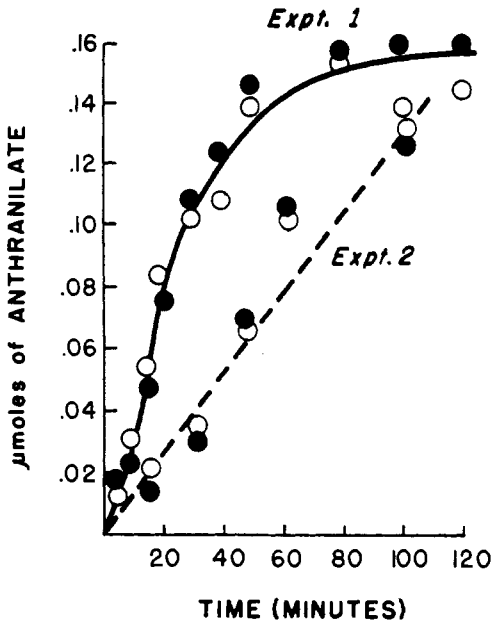

FIG. 1.—Effect of PEP on the synthesis of anthranilate. Expt. 1: The reaction mixture consisted of 0.5 μmole of shikimate 5-phosphate, 0.5 μmole of L-glutamine, 50 μmoles of Tris buffer, *p*H 8.2, 5 μmoles of $MgCl_2$, 0.5 μmole of NAD, 0.5 μmole of PEP, where indicated, and 0.1 ml of crude *E. coli* B-37 extract (2 mg of protein) in a final volume of 1 ml. The reaction mixture was incubated at 37°, ●—●, in the presence of PEP and O—O, in the absence of PEP. Expt. 2: The procedure was the same as in experiment 1 except that a different preparation of *E. coli* B-37 extract was used.

Fractionation of E. coli B-37 Extracts into Two Enzyme Systems.—Method A. $(NH_4)_2SO_4$ Fractionation.—Twenty ml of crude *E. coli* B-37 extract was fractionated into 0–30%, 30–50%, and 50–75% saturation fractions by the addition of 3.5 g, 2.2 g, and 2.9 g of powdered $(NH_4)_2SO_4$ sequentially. The precipitates obtained after each addition of $(NH_4)_2SO_4$ were removed by centrifugation, dissolved in 10 ml of Tris buffer (0.033 M, *p*H 7.4), and dialyzed against 3 liters of this buffer for a total of 5 hours with two changes of buffer.

Method B. Protamine Sulfate Fractionation.—To 470 ml of *E. coli* B-37 extract (18 mg of protein per ml) was added dropwise 54 ml of a 2% protamine sulfate solution. The suspension was stirred for 30 minutes and followed by centrifugation at 15,000 × *g*. The supernatant from this centrifugation was designated as *protamine supernatant.*

The precipitate obtained in the above centrifugation was suspended in 200 ml of 0.2 M Tris buffer, *p*H 9.0, with the aid of a glass homogenizer. The resulting suspension was stirred for 30 minutes, and the undissolved residue was removed by centrifugation at 15,000 × *g* and discarded. The supernatant was adjusted to *p*H 7.0 with 1 N acetic acid and was stored at −20°. This supernatant (6 mg of protein per ml) was designated *protamine extract.*

$(NH_4)_2SO_4$ *Fractionation of the Protamine Supernatant.*—To 500 ml of the protamine supernatant solution was added 119 g of powdered ammonium sulfate, slowly with stirring, bringing the solution to a level of 40% saturation. After an additional 30 minutes' stirring the precipitate was removed by centrifugation and discarded. The supernatant was brought to 60% saturation by the addition of 59 g of powdered ammonium sulfate. The precipitate was removed by centrifugation and dissolved in 50 ml of 0.033 M Tris buffer, *p*H 7.4, containing reduced glutathione (10^{-4} M) and Versene (10^{-4} M). The solution was dialyzed against 3 liters of the above buffer for 4.5 hours with two changes of buffer. The resulting enzyme solution (26 mg of protein per ml) was again fractionated with solid $(NH_4)_2SO_4$. The precipitate obtained between the 40% and 60% saturated ammonium sulfate fraction was dissolved in 40 ml of buffer and dialyzed as indicated above. This enzyme fraction (20 mg of protein per ml) was designated as partially purified fraction I of *E. coli* B-37 extracts.

$(NH_4)_2SO_4$ *Fractionation of the Protamine Extract.*—During storage of the protamine extract at −20° for a few days a precipitate was formed. This was removed by centrifugation at 15,000 × *g* and discarded. To 190 ml of the supernatant (6 mg of protein per ml) was added slowly with stirring 39.0 g of powdered ammonium sulfate, to bring the solution to 30% saturation. After 30 minutes' stirring the precipitate was removed by centrifugation at 15,000 × *g* and dissolved in 60 ml of 0.033 M Tris buffer, *p*H 7.4, containing reduced glutathione (10^{-4} M) and Versene (10^{-4} M). The resulting solution (17 mg of protein per ml) was dialyzed against 3 liters of the above buffer for a total of 4.5 hours with two changes of buffer. The ammonium sulfate fractionation was repeated and the resulting precipitate (0–30% saturation) was dissolved in 40 ml of the above buffer and dialyzed as before. The dialyzed solution (13 mg of protein per ml) was stored at −20°. During storage a precipitate was formed which was resuspended in the enzyme solution for use in the experiments described, since removal of the precipitate by centrifugation caused a loss of some enzymatic activity. This preparation was designated as partially purified fraction II of *E. coli* B-37 extract. All the above operations were carried out at 2°.

Microbiological Assay of Shikimic Acid and Its Derivatives.—Shikimate was determined by microbiological assay using *A. aerogenes* mutant A170-143 S1 (Davis and Weiss, 1953). Shikimate-5-P was estimated microbiologically after its conversion to shikimate by potato acid phosphatase (Weiss and Mingioli, 1956). 3-Enolpyruvylshikimate was hydrolyzed with acid to shikimate and then assayed microbiologically (Davis and Mingioli, 1953). ESP was converted to shikimate by treatment with alkaline phosphatase followed by acid hydrolysis; the released shikimate was a measure of ESP in the reaction mixture.

Anthranilate was determined by the colorimetric method of Bratton and Marshall (1939) as modified by Eckert (1943). Inorganic phosphate was estimated by the method described by Bruemmer and O'Dell (1956). The protein content of the enzyme solutions was analyzed by the method of Warburg and Christian (1941–42).

RESULTS

Studies with Crude Extracts of E. coli B-37.—Since the formation of ESP requires the condensation of shikimate-5-P and PEP, the synthesis of anthranilate from shikimate-5-P and L-glutamine was examined for stimulation by PEP with cell-free extracts of *E. coli* B-37. The results clearly indicated (Fig. 1) that PEP enhanced neither the rate nor the total amount of synthesis appreciably.

The synthesis of anthranilate from shikimate-5-P and L-glutamine was found to be inhibited by fluoride and by phosphate. Whereas fluoride abolished the synthesis completely at a concentration of 5 μmoles per ml, phosphate inhibited the synthesis only at relatively high concentrations: with phosphate, 50% inhibition was obtained at 30 μmoles per ml of reaction mixture. However, the inhibition by both fluoride

TABLE I

REVERSAL OF THE FLUORIDE AND PHOSPHATE INHIBITIONS OF ANTHRANILATE SYNTHESIS

The reaction mixture consisted of 0.5 μmole of shikimate 5-phosphate, 2.5 μmoles of L-glutamine, 50 μmoles of Tris buffer, *p*H 8.2, 5 μmoles of $MgCl_2$, 0.5 μmole of NAD, 150 μmoles of ethanol, 0.15 mg of alcohol dehydrogenase, 0.1 ml of crude *E. coli* B-37 extract (2.0 mg of protein) plus additions, in a final volume of 1 ml. The reaction mixture was incubated at 37° for 90 minutes. PEP, fluoride, and phosphate, when added, were at concentrations of 0.5, 20, and 40 μmoles, respectively, per ml.

Additions	Anthranilate Formed (μmole)
None	0.37
PEP	0.34
Fluoride	0.04
Fluoride + PEP	0.29
Phosphate	0.12
Phosphate + PEP	0.30

and phosphate could be easily reversed by PEP (Table I). This suggested that PEP could be involved in the formation of anthranilate from shikimate-5-P and L-glutamine, although the earlier studies indicated that PEP did not stimulate anthranilate synthesis. These facts would be reconciled if PEP were being formed from some compound added to the reaction mixture. L-glutamine seemed to be the most logical source.

Formation of PEP from L-Glutamine by E. coli B-37 Extracts.—Preliminary experiments indicated that extracts of *E. coli* B-37 contained the enzymes DAHP synthetase (Srinivasan and Sprinson, 1959) and 5-dehydroquinate synthetase (Sprinson *et al.*, 1962) which convert erythrose 4-phosphate and PEP to 5-dehydroquinate via 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP). If L-glutamine could replace PEP in these reactions and give rise to DAHP and 5-dehydroquinate, then the speculation that L-glutamine was being converted to PEP would be justified. The results represented in Figure 2 and Table II establish that L-glutamine can substitute for PEP in the synthesis of DAHP and 5-dehydroquinate from erythrose 4-phosphate with B-37 extracts. These observations also explain the inability of PEP to stimulate the synthesis of anthranilate from shikimate-5-P and L-glutamine.

The demonstration of the synthesis of PEP from L-glutamine increased the plausibility of the suggestion that ESP may be an intermediate in the anthranilate pathway. To explore this idea, cell-free extracts of *E. coli* B-37 were incubated with ESP and L-glutamine. Under these conditions the disappearance of ESP was accompanied by the appearance of *both* shikimate-5-P and anthranilate. Since these extracts are capable of converting shikimate-5-P and L-glutamine to anthranilate, it was difficult to differentiate between the following situations: (a) a part of the ESP was being converted to anthranilate directly, or (b) ESP was first transformed to shikimate-5-P which then participated as a carbon source. To surmount this difficulty, fractionation of the cell-free extracts was resorted to with a view to removing the enzymes involved in the conversion of ESP to shikimate-5-P and also of glutamine to PEP.

Studies with Partially Purified Enzyme Preparations.—Fractionation of crude extracts of *E. coli* B-37 with $(NH_4)_2SO_4$ (method A) resulted in the separation of the anthranilate-forming enzyme system into two distinct fractions (*i.e.*, 0–30% and 30–50% $(NH_4)_2SO_4$ fractions). Each fraction alone did not synthesize anthranilate from shikimate-5-P and L-glutamine (Table III). However, a combination of 0–30 and 30–50% fractions resulted in anthranilate synthesis. Furthermore, the substrate shikimate-5-P disappeared in the presence of one of the fractions (*i.e.*, 30–50%), although no anthranilate was produced. This fraction was therefore considered to possess the earlier enzymes required for the synthesis of anthranilate (*i.e.*, fraction

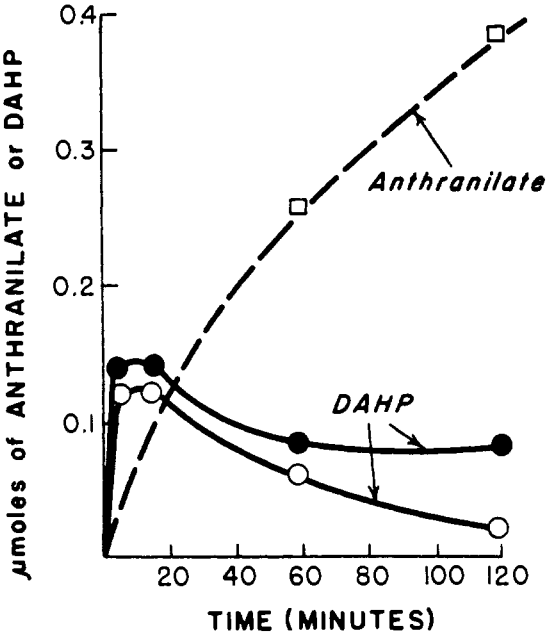

FIG. 2.—Conversion of L-glutamine to PEP as indicated, by the synthesis of DAHP. O—O, Synthesis of DAHP from 0.49 μmole erythrose 4-phosphate, 0.6 μmole of PEP, 5 μmoles of $MgCl_2$, 50 μmoles of glycine buffer, *p*H 7.4, and 0.1 ml crude *E. coli* B-37 extract (2 mg of protein) in a final volume of 1 ml. ●—●, Synthesis of DAHP from 2.5 μmoles of L-glutamine, 2.8 μmoles of erythrose 4-phosphate, 0.5 μmole of NAD, 5 μmoles of $MgCl_2$, B-37 extract (2 mg of protein) in a final volume of 1 ml. □---□, Synthesis of anthranilate from 2.5 μmoles of L-glutamine, 0.5 μmole of shikimate-5-P, 5 μmoles of $MgCl_2$, 0.5 μmole of NAD, 50 μmoles of glycine buffer, *p*H 7.4, and 0.1 ml crude *E. coli* B-37 extract (2 mg of protein) in a final volume of 1 ml. All incubations were carried out at 37°.

TABLE II

CONVERSION OF L-GLUTAMINE TO PEP AS DETERMINED BY THE ENZYMATIC SYNTHESIS OF 5-DEHYDROQUINATE

The incubation mixture consisted of 0.5 μmole of NAD, 50 μmoles of glycine buffer, *p*H 7.4, 5 μmoles of $MgCl_2$, 0.5 μmole of $CoSO_4$, the substrates indicated below, and 0.2 ml of crude *E. coli* B-37 extract (4 mg of protein), in a final volume of 1 ml. The reaction mixture was incubated at 37° for 1 hour. 5-Dehydroquinate was estimated by microbiological assay with *A. aerogenes* mutant A170-143S1 (Davis and Weiss, 1953).

Substrate	5-Dehydroquinate Formed (μmole) Exp. 1	Exp. 2
0.6 μmole of PEP + 0.49 μmole of erythrose-4-P	0.12	0.11
2.5 μmoles of L-glutamine + 2.8 μmoles of erythrose-4-P	0.26	0.21
2.5 μmoles of L-glutamine	0	0
2.8 μmoles of erythrose-4-P	0	0

TABLE III

ENZYMATIC ACTIVITY OF THE $(NH_4)_2SO_4$ FRACTIONS OF *E. coli* B-37 EXTRACTS

The reaction mixture consisted of 0.69 μmole of shikimate 5-phosphate, 2.5 μmoles of L-glutamine, 2.0 μmoles of $MgCl_2$, 100 μmoles of Tris buffer, *p*H 8.2, 0.5 μmole of NAD, and 0.1 ml of the enzyme fraction (1.5 mg each of protein) indicated, in a final volume of 1 ml. The reaction was incubated at 37° for 2 hours.

$(NH_4)_2SO_4$ Fraction	Shikimate-5-P Disappeared (μmole)	Anthranilate Formed (μmole)
0–30%	0.09	0.01
30–50%	0.68	0
0–30% plus 30–50%	—	0.18

TABLE IV

RECONSTITUTION OF PROTAMINE SULFATE AND $(NH_4)_2SO_4$ FRACTIONATED *E. coli* B-37 EXTRACTS FOR ANTHRANILATE SYNTHESIS

The reaction mixture consisted of 0.5 μmole of shikimate-5-P, 2.5 μmoles of L-glutamine, 0.5 μmole of NAD, 4 μmoles of $MgCl_2$, 20 μmoles of Tris buffer, *p*H 8.2, and 0.1 ml of the indicated extracts (1.5 mg of protein), in a final volume of 1 ml. The mixture was incubated at 37° for 2 hours.

Enzyme Fractions	Anthranilate Formed (μmole)
Crude *E. coli* B-37 extract	0.33
0–30% $(NH_4)_2SO_4$	0
30–50% $(NH_4)_2SO_4$	0
Protamine supernatant	0
Protamine extract	0
Protamine supernatant + protamine extract	0.23
Protamine supernatant + 0–30% $(NH_4)_2SO_4$	0.19
Protamine supernatant + 30–50% $(NH_4)_2SO_4$	0

I). The 0–30% $(NH_4)_2SO_4$ fraction therefore contained the later enzymes in this pathway (fraction II).

The protamine sulfate treatment of the crude extracts of *E. coli* B-37 for the removal of nucleic acids (method B) also brought about its fractionation into two enzyme systems.[2] The protamine supernatants did not synthesize anthranilate from shikimate-5-P and L-glutamine (Table IV). However, the synthesis could be restored either by the addition of the protamine extract or by addition of the 0–30% $(NH_4)_2SO_4$ fraction prepared by method A. These studies suggested that protamine sulfate treatment of *E. coli* B-37 extracts yielded a fractionation pattern similar to that obtained by $(NH_4)_2SO_4$ fractionation. Thus the protamine supernatant behaved as the 30–50% $(NH_4)_2SO_4$ fraction and therefore contains the earlier enzymes of the anthranilate pathway.

Enzyme Complementation Studies.—The separation of the crude extracts into two distinct fractions facilitated "enzyme complementation" studies with extracts of *A. aerogenes* mutants[3] A170-40 and A170-44. Neither of these extracts individually could form anthranilate from shikimate-5-P and L-glutamine, but in combination with each other or with *E. coli* B-37 protamine supernatant synthesis of anthranilate was observed (Table V).

TABLE V

ENZYME COMPLEMENTATION STUDIES

The reaction mixture consisted of 0.5 μmole of shikimate 5-phosphate, 2.5 μmoles of L-glutamine, 50 μmoles of Tris buffer, *p*H 8.2, 5 μmoles of $MgCl_2$, 0.5 μmole of NAD, and 0.1 ml of extracts indicated below (2 mg of protein), in a final volume of 1 ml. The reaction mixture was incubated at 37° for 2 hours.

Enzyme Extracts[a]	Anthranilate Formed (μmole)
Protamine supernatant + A170-40	0.10
Protamine supernatant + A170-44	0.13
Protamine extract + A170-40	0
Protamine extract + A170-44	0
A170-40 + A170-44	0.26
Protamine extract + try A-2	0.19
Protamine extract + try A-6	0.14
Protamine extract + try A-8	0.19
Protamine supernatant + try A-2	0
Protamine supernatant + try A-6	0
Protamine supernatant + try A-8	0
Try A-2 + try A-6 + try A-8	0

[a] None of the extracts synthesized anthranilate when incubated alone under the conditions indicated.

Similar enzyme complementation studies were also carried out with the extracts of *S. typhimurium* mutants[3] try A-2, try A-6, and try A-8 (Table V). These extracts did not synthesize anthranilate from shikimate-5-P and L-glutamine. However, anthranilate was formed when the extracts of the mutants were combined with protamine extract of *E. coli* B-37 or 0–30% $(NH_4)_2SO_4$ fraction. It can also be seen that the extracts of these three *Salmonella* mutants and B-37 protamine supernatant were lacking in at least one common enzyme since they did not complement each other.

Role of 3-Enolpyruvylshikimate 5-Phosphate in the Synthesis of Anthranilate.—The enzyme fractions obtained from the protamine sulfate treatment of *E. coli* B-37 extracts were fractionated further by subjecting them to two ammonium sulfate fractionations. When combined, partially purified fractions I and II of *E. coli* B-37 extracts could no longer synthesize anthranilate from shikimate-5-P, L-glutamine, and NAD. However it was found that the addition of PEP, reduced glutathione, and a NADH-regenerating system to the reaction mixture restored the synthesis (Table VI).

TABLE VI

SYNTHESIS OF ANTHRANILATE FROM 3-ENOLPYRUVYLSHIKIMATE-5-P BY PARTIALLY PURIFIED ENZYME FRACTIONS I AND II

The reaction mixture contained 0.5 μmole of L-glutamine, 5 μmoles of $MgCl_2$, 5 μmoles of reduced glutathione, 50 μmoles of Tris buffer, *p*H 8.2, 0.5 μmole of NAD, 150 μmoles of ethanol, 0.15 mg alcohol dehydrogenase, 0.1 ml each of purified enzyme fractions I and II (total protein 2.36 mg), and 0.5 μmole of the indicated substrates in a total volume of 1 ml. The mixture was incubated for 2 hours at 37°.

Additions	Anthranilate Synthesized (μmole)
None	0
Shikimate-5-P	0.01
Shikimate-5-P + PEP	0.25
Shikimate-5-P + pyruvate	0.01
3-Enolpyruvylshikimate-5-P	0.24

[2] Occasionally the protamine extract and the protamine supernatant had some residual capacity to catalyze the complete synthesis. This was readily removed by the first $(NH_4)_2SO_4$ fractionation as described under Experimental.

[3] *A. aerogenes* A170-40 is blocked between shikimate-5-P and ESP, and A170-44 is blocked immediately after ESP. The *Salmonella* mutants try A-2, try A-6, and try A-8 accumulate shikimate and shikimate-5-P in their growth media and are blocked between the latter and anthranilate.

TABLE VII

SYNTHESIS OF 3-ENOLPYRUVYLSHIKIMATE-5-P BY PARTIALLY PURIFIED ENZYME FRACTION I

The reaction mixture consisted of 0.5 μmole of shikimate 5-phosphate, 50 μmoles of Tris buffer, *p*H 8.2, 5 μmoles of $MgCl_2$, 0.5 μmole of NAD, 150 μmoles of ethanol, 0.15 mg of alcohol dehydrogenase, and 0.5 μmole of the additions as indicated below, and 0.1 ml of partially purified enzyme fraction I of *E. coli* B-37 extract (1.3 mg of protein) in a final volume of 1 ml. The reaction mixture was incubated for 30 minutes at 37°.

Addition	3-Enolpyruvyl-shikimate-5-P Formed (μmole)
None	0.02
PEP	0.17

Shikimate-5-P and PEP could be replaced by their enzymatic condensation product, ESP. PEP could not be replaced by pyruvate.

These results indicate that ESP is an intermediate in the biosynthetic pathway from shikimate-5-P to anthranilate. The efficient conversion of shikimate-5-P and PEP to anthranilate implies that these fractions contain the synthetase necessary for the formation of ESP from these two substrates. This has been confirmed by the demonstration that fraction I can carry out the condensation reaction (Table VII).

Further confirmation of the role of ESP in anthranilate biosynthesis was obtained by investigating the behavior of extracts of A170-40 and A170-44 toward different substrates. A170-40 accumulates shikimate-5-P (Davis and Mingioli, 1953) in the medium and is blocked between shikimate-5-P and ESP. A170-44 accumulates 3-enolpyruvylshikimate (Davis and Mingioli, 1953) and a little ESP, and is blocked immediately after the latter compound.

Extracts of A170-44 synthesized negligible amounts of anthranilate from either shikimate-5-P + PEP or ESP in the presence of glutamine (Table VIII).

Unlike extracts of A170-44, those of A170-40 can form anthranilate from ESP and glutamine. However, shikimate-5-P and PEP cannot substitute for ESP. These results are in complete agreement with the mutational block assigned to these mutants from growth requirements and accumulation studies. Thus, these enzyme studies with the extracts also support the conclusion that ESP is an intermediate in the biosynthesis of anthranilate.

Requirements for Anthranilate Synthesis.—The conversion of shikimate-5-P, PEP, and L-glutamine to anthranilate by partially purified fractions of *E. coli* B-37 required the presence of a NADH-regenerating system (Table IX). NAD was also required if NADP and a NADPH-regenerating system were substituted in lieu of the NADH-regenerating system.

L-Glutamine was also the most efficient amino donor for anthranilate synthesis from ESP. Although in the

TABLE VIII

BEHAVIOR OF EXTRACTS OF *Aerobacter aerogenes* MUTANTS TOWARDS VARIOUS SUBSTRATES

The reaction mixture contained 2.5 μmoles of L-glutamine, 50 μmoles of Tris buffer, *p*H 8.2, 5 μmoles of $MgCl_2$, 5 μmoles of reduced glutathione, 0.5 μmole of NAD, 150 μmoles of ethanol, 0.15 mg of alcohol dehydrogenase, 0.1 ml of enzyme extract (2 mg protein), and 0.5 μmole of the indicated substrates in a total volume of 1 ml. The reaction mixture was incubated for 2 hours at 37°.

Substrates	Enzyme Extract	Anthranilate Synthesized (μmole)
Shikimate-5-P + PEP	A170-40	0.01
Shikimate-5-P + PEP	A170-44	0.01
3-Enolpyruvylshikimate-5-P	A170-40	0.31
3-Enolpyruvylshikimate-5-P	A170-44	0.03

TABLE IX

COFACTOR REQUIREMENTS FOR ANTHRANILATE SYNTHESIS BY PARTIALLY PURIFIED FRACTIONS I AND II

The reaction mixtures consisted of 0.5 μmole each of shikimate 5-phosphate, PEP, and L-glutamine, 50 μmoles of Tris buffer, *p*H 8.2, 5 μmoles of $MgCl_2$, 5 μmoles of reduced glutathione, and 0.1 ml each of the partially purified fractions I and II (0.32 mg of protein) in a final volume of 1 ml. The mixture was incubated for 90 minutes at 37°. Cofactors were added at 0.5-μmole levels.

Additions	Anthranilate Formed (μmole) Exp. 1	Exp. 2
None	0	0
NAD	0	0
NADP	0	0
NADP, NAD	0	0.02
NAD, NADH-regenerating system[a]	0.22	0.17
NAD, NADP, NADPH-regenerating system[b]	0.12	0.11
NADP, NADPH-regenerating system[b]	0	0.02
NADP, NAD, NADH-regenerating system[a]	0.20	0.20
NAD, ATP, ATP-regenerating system[c]	0	0

[a] 150 μmoles of ethanol and 0.15 mg of alcohol dehydrogenase. [b] 5 μmoles of glucose-6-P and 0.05 ml of glucose-6-P dehydrogenase (0.06 mg protein). [c] 5 μmoles of creatine-P and 0.09 mg creatine-P transphosphorylase.

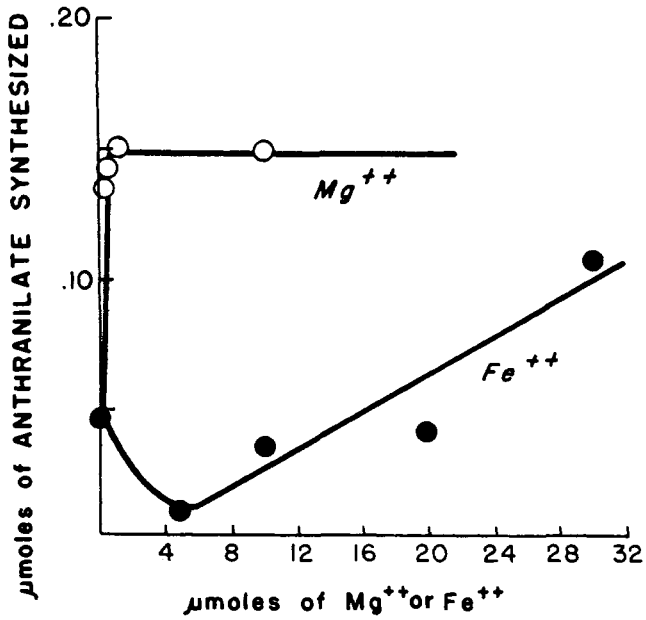

FIG. 3.—Effect of Mg^{++} and Fe^{++} on the synthesis of anthranilate. The reaction mixture consisted of 0.5 μmole of shikimate-5-P, 0.5 μmole of PEP, 0.5 μmole of L-glutamine, 50 μmoles of Tris buffer, *p*H 8.2, 0.5 μmole of NAD, 150 μmoles of ethanol, 0.15 mg of alcohol dehydrogenase, 5 μmoles of reduced glutathione, and 0.1 ml each of partially purified fractions I and II of *E. coli* B-37 extracts (0.32 mg of protein) in a final volume of 1 ml. The mixture was incubated at 37° for 2 hours. O—O, Mg^{++}; ●—●, Fe^{++}.

TABLE X

CONDITIONS NECESSARY FOR THE RELEASE OF PHOSPHATE FROM 3-ENOLPYRUVYLSHIKIMATE-5-P BY PARTIALLY PURIFIED FRACTIONS OF *E. coli* B-37

The reaction mixture consisted of 0.5 μmole of ESP, 50 μmoles of Tris buffer, *p*H 8.2, 5 μmoles of $MgCl_2$, 5 μmoles of reduced glutathione, 150 μmoles of ethanol, 0.15 mg of alcohol dehydrogenase, and 0.1 ml each of partially purified fractions I and II (1.5 mg of protein) and additions in a final volume of 1 ml. (L-Glutamine and NAD, when added, were at a concentration of 2.5 and 0.5 μmoles, respectively, per ml.) The reaction mixture was incubated at 37° for 20 minutes.

Additions	Phosphate Released (μmole)	Anthranilate Formed (μmole)
L-Glutamine	0.01	0
NAD	0.17	0
L-Glutamine + NAD	0.17	0.18

crude extracts Mg^{++} or Fe^{++} satisfied the metal requirement (Srinivasan and Rivera, 1963), the partially purified fractions I and II gave far better anthranilate synthesis with low concentrations of Mg^{++} than with Fe^{++} (Fig. 3).

Compound X, a New Intermediate in the Biosynthesis of Anthranilate.—The results presented in Tables IV to VIII also permit the postulation of another intermediate in this pathway. Extracts of A170-44 and enzyme fraction I by themselves cannot utilize ESP for anthranilate synthesis but do so when combined. Similarly, fraction II cannot form anthranilate from ESP but the addition of fraction I does result in anthranilate synthesis. Furthermore, ESP does not disappear in the presence of fraction II. It follows therefore that fraction I must convert ESP to a new compound which is then utilized by A170-44 extract or by fraction II to form anthranilate.

Fraction I was found to release inorganic phosphate from ESP in the presence of a NADH-regenerating system alone (Table X). Under these conditions no anthranilate was synthesized. In order to determine whether any intermediate between ESP and anthranilate was being formed, ESP was incubated with partially purified fraction I in the absence of L-glutamine (reaction A). Aliquots of the incubation mixture were removed at different intervals and placed in a boiling water bath for 3 minutes to arrest the enzymatic reaction. One portion of the various aliquots was used for the estimation of phosphate and anthranilate. The other portion was incubated in the presence of L-glutamine and *A. aerogenes* A170-44 extract (reaction B). Aliquots of the second reaction mixture were assayed for anthranilate. Anthranilate was produced *only* during the second incubation (Fig. 4). Since neither *A. aerogenes* A170-44 extracts nor partially purified fraction II of B-37 could synthesize anthranilate from ESP, these observations indicate that a new compound, capable of being converted to anthranilate, was formed from ESP by partially purified fraction I in the absence of L-glutamine.

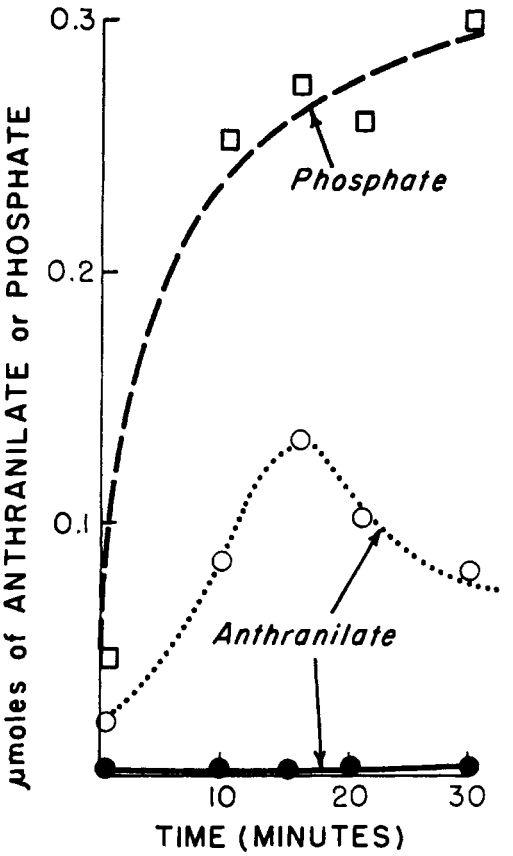

FIG. 4.—Evidence for the conversion of ESP to 'compound X.' Reaction A consisted of 0.5 μmole of 3-enolpyruvylshikimate 5-phosphate, 50 μmoles of Tris buffer, *p*H 8.2, 5 μmoles of $MgCl_2$, 0.5 μmole of NAD, 5 μmoles of reduced glutathione, 150 μmoles of ethanol, 0.15 mg of alcohol dehydrogenase, 0.2 ml of partially purified fraction I (1.51 mg of protein) in a final volume of 0.65 ml. The reaction mixture was incubated at 37°. The reaction was stopped by placing it in a boiling water bath for 3 minutes. Reaction B consisted of 0.65 ml of reaction A, 2.5 μmoles of L-glutamine, 50 μmoles of Tris buffer, *p*H 8.2, 5 μmoles of $MgCl_2$, 0.5 μmole of NAD, 5 μmoles of reduced glutathione, 150 μmoles of ethanol, 0.15 mg of alcohol dehydrogenase, 0.1 ml of *A. aerogenes* A170-44 extract (2 mg of protein) in a final volume of 1 ml. The reaction mixture was incubated at 37° for 2 hours. □---□, Phosphate released during reaction A; ●—●, anthranilate synthesized during reaction A; O···O, anthranilate synthesized from 'compound X' in reaction B.

DISCUSSION

The results presented here establish 3-enolpyruvylshikimate 5-phosphate as an intermediate in the biosynthesis of anthraniiate. This has been accomplished by demonstrating: (a) the conversion of ESP to anthranilate in fractionated extracts of *E. coli* B-37 which can no longer utilize the earlier precursor, shikimate-5-P; (b) the presence in these extracts of the enzyme ESP synthetase which catalyzes the synthesis of ESP from shikimate-5-P and PEP; (c) the conversion of ESP to another compound which is a precursor of anthranilate.

This conclusion has been further confirmed by the finding that extracts of mutants which lack ESP synthetase (*A. aerogenes* A170-40) did not synthesize anthranilate from shikimate-5-P, PEP, and L-glutamine, although they converted ESP to anthranilate in the presence of L-glutamine. Extracts of mutants which are blocked immediately after ESP (*A. aerogenes* A170-44) cannot utilize this compound or shikimate-5-P and PEP for anthranilate synthesis. These observations fulfill the minimum necessary criteria for establishing a compound as an intermediate in a metabolic pathway. These conclusions are also in complete agreement with the accumulation studies and the nutritional requirements of the various aromatic amino acid mutants. Recently, Gibson *et al.* (1962), on the basis of indirect evidence, have also implicated the participation of ESP in the biosynthesis of anthranilate in *A. aerogenes*.

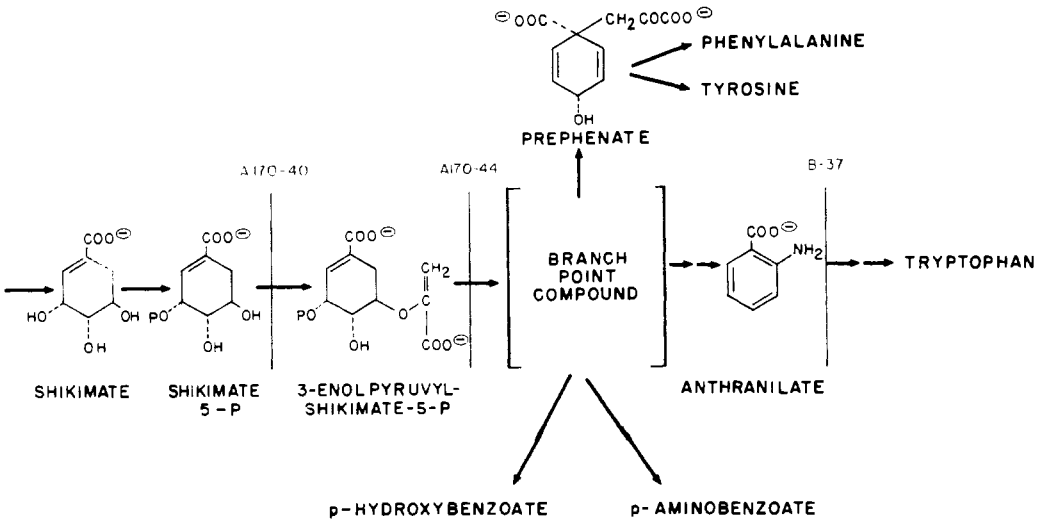

Fig. 5. Scheme for the biosynthesis of aromatic compounds.

The efficient conversion of shikimate-5-P to anthranilate in the presence of L-glutamine and NAD by crude extracts is due to the formation of PEP (which is necessary for this reaction) from the enzymatic degradation of L-glutamine. This has been demonstrated by trapping the PEP formed from glutamine with erythrose 4-phosphate to yield DAHP and 5-dehydroquinate. The synthesis of anthranilate also requires the presence of a NADH-regenerating system and Mg^{++}.

The occurrence of mutants (Davis and Mingioli, 1953) accumulating 3-enolpyruvylshikimate in their media and requiring supplementation with phenylalanine, tyrosine, tryptophan, *p*-aminobenzoate, and *p*-hydroxybenzoate for growth suggests the existence of a common intermediate after ESP. Henceforth this intermediate will be referred to as *Branch Point Compound.*

From the enzyme complementation studies it is evident that extracts of *S. typhimurium* mutants try A-2, try A-6, and try A-8, and *E. coli* B-37 enzyme fraction I, are missing at least one enzyme in common since they could not supplement each other in synthesizing anthranilate. The exact mutational block of the *S. typhimurium* try A-2, try A-6, and try A-8 mutants is not known, except that it must be beyond the Branch Point Compound which is common to the anthranilate, prephenate, *p*-aminobenzoate, and *p*-hydroxybenzoate pathways. On the other hand, *A. aerogenes* A170-44 extract and *E. coli* B-37 enzyme fraction II contain all the enzymes necessary for anthranilate synthesis beyond the one which converts ESP to the next intermediate. Consequently, compound X, which was formed by *E. coli* B-37 enzyme fraction I in the presence of NADH, could be either the Branch Point Compound or some compound between this and anthranilate.

The nature of the new intermediate is under current investigation in our laboratory. Recently, preliminary evidence has been presented by Gibson and Gibson (1962) for the synthesis of a compound from shikimate, ATP, NADH, and ribose 5-phosphate by crude extracts of a triple auxotroph of *A. aerogenes.* This compound was found to be converted to anthranilate and to prephenate by the appropriate enzyme systems. It has not been identified, but it may well be the Branch Point Compound.

On the basis of both the present work and the recent observations of Clark and Sprinson concerning the formation of prephenate, the scheme shown in Figure 5, for the biosynthesis of aromatic amino acids can be advanced. The Branch Point Compound probably has the enolpyruvyl group intact so that it can rearrange to form prephenate. This compound should also be capable of forming anthranilate, *p*-hydroxybenzoate, and *p*-aminobenzoate.

Acknowledgments

We are extremely grateful to Drs. B. D. Davis, M. Demerec, and J. S. Gots for the mutant strains used in this investigation, and to Drs. M. J. Clark and D. B. Sprinson for providing us with a generous sample of 3-enolpyruvylshikimate 5-phosphate for the initial exploratory experiments.

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