The Enzymatic Synthesis of Anthranilate from Shikimate 5-Phosphate and L-Glutamine*

P. R. SRINIVASAN AND AMERICO RIVERA, JR.†

From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York

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Shikimate 5-phosphate is converted to anthranilate by cell-free extracts of *E. coli* mutant B-37 in the presence of L-glutamine. A NADH regenerating system and either Mg^{++} or Fe^{++} are required for this process. It is shown that the amino group of anthranilate is derived from the amide-N of L-glutamine. This is in accord with the inhibitory effects observed with the structural analogs of glutamine.

Nutritional studies with bacterial mutants have indicated that 5-dehydroquinate (Weiss *et al.*, 1953), 5-dehydroshikimate (Salamon and Davis, 1953) and shikimate (Davis, 1951) are successive intermediates in the biosynthesis of several aromatic amino acids and vitamins. The occurrence of certain multiple aromatic auxotrophs of *Escherichia coli*, *Salmonellae* species, and *Aerobacter* species which accumulated compounds "Z1" and "Z2" in their media suggested that these compounds could also participate as intermediates in the biosynthesis of aromatic amino acids (Davis and Mingioli, 1953). Compound Z2 has been identified as shikimate 5-phosphate (Weiss and Mingioli, 1956) and Z1 has been identified as the 3-enolpyruvyl ether of shikimate (Borowitz and Sprinson, unpublished observations). Compound Z1 has also been shown to be formed from shikimate-5-P and phosphoenolpyruvate in cell-free extracts of *E. coli* (Kalan and Davis, unpublished observations).[1] In this paper evidence is presented for the synthesis of anthranilate (an intermediate in the biosynthesis of tryptophan) from shikimate-5-P and L-glutamine in extracts of *E. coli* B-37. The amino group of anthranilate is shown to be derived from the amide-N of L-glutamine. The cofactor requirements of the system have also been studied. A part of these results have appeared in preliminary form (Srinivasan, 1959).

Experimental Procedure

Materials.—Shikimate-5-P was isolated from the culture medium of *A. aerogenes* strain A170-40 as the barium salt (Weiss and Mingioli, 1956) and converted to the potassium salt for the enzymatic experiments. The two isomers of 6-amino-3,4,5-trihydroxycyclohexane carboxylic acid were kindly provided by Professor H. Plieninger, Heidelberg. L-Glutamine-(amide-N^{15}) was a generous gift of Dr. A. Neidle. Adenosine-phosphoramidate was a generous gift of Dr. R. Chambers. *Neurospora* nicotinamide adenine dinuclease (60% acetone fraction) was kindly provided by Dr. N. O. Kaplan. Aza-L-serine and 6-diazo-5-oxo-L-norleucine were kindly given to us by Dr. E. Borek. *O*-carbazyl-DL-serine was a generous gift of Dr. W. Shrive and, γ-glutamylhydrazine and diazoacetic ester of 6-hydroxynorleucine were generously provided by Dr. Alexander Moore of the Mellon Institute and Parke, Davis and Company.

Preparation of Enzyme Extract.—*Escherichia coli* mutant B-37 (blocked between anthranilate and indoleglycerol 3′-phosphate) was grown for 18 hours with aeration at 30° in medium A (Davis and Mingioli, 1950) supplemented either with 5 μg of tryptophan per ml or with 0.2% yeast extract (Difco) and 0.2% casein hydrolysate. The cells were harvested by centrifugation at 2°, washed with cold water, suspended in 0.033 M phosphate buffer, *p*H 7.4 (20 ml/5.0 g of wet bacteria), and disrupted by means of sonic oscillation for 30 minutes in a 9-kc Raytheon oscillator cooled with circulating ice water. Centrifugation at 12,000 × *g* in a Lourdes preparative centrifuge yielded a clear, pale yellow solution containing 20 mg of protein per ml. At −15° it retained adequate activity over a period of 6 months.

Pyridine nucleotides were removed from certain preparations by treatment with charcoal (1 mg per mg of protein at 0°). The mixture was stirred every 5 minutes by gentle aspiration and release from a pipet. After 30 minutes the charcoal was removed by centrifugation at 0°.

Assay of Anthranilate.[2]—Samples of the incubation mixture (0.5 ml), taken at zero time and after stated intervals were deproteinized with 0.2 ml of 10% trichloroacetic acid and the mixture was centrifuged. Anthranilic acid in the supernatant solution was estimated by the method of Bratton and Marshall (1939) as modified by Eckert (1943). The purple color produced was measured at 550 mμ in a spectrophotometer.

Conversion of Shikimate-5-P and Glutamine-(amide-N^{15}) to Anthranilate.—Fifty μmoles of shikimate-5-P, 2000 μmoles of Tris buffer, *p*H 8.2, 100 μmoles of $MgCl_2$, 100 μmoles of glutamine-(amide-N^{15}) and 10 ml of crude enzyme extract in a total volume of 50 ml were incubated for 3 hours at 37°. After incubation, the reaction mixture was inactivated with 1.0 ml of 6 N H_2SO_4. The precipitated protein was removed by centrifugation. The precipitate was washed with water and the washings were added to the supernatant. Total volume was 55.0 ml. The anthranilic acid in the supernatant was assayed in triplicate, at three levels, by the colorimetric method. To 50 ml of the supernatant solution which contained 20.9 μmoles (2.86 mg) of anthranilic acid, 100 mg of carrier anthranilic acid was added. The anthranilic acid was then isolated by continuous ether extraction and was recrystallized from H_2O and sublimed *in vacuo*. It was then analyzed for N^{15} by standard methods.

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[1] The following abbreviations have been used: shikimate-5-P, shikimate 5-phosphate; DON, 6-diazo-5-oxo-L-norleucine.

[2] The reaction mixtures were also assayed microbiologically with appropriate mutants blocked before and after anthranilate. The results agreed with the values obtained by the colorimetric method.

TABLE I

SYNTHESIS OF ANTHRANILATE FROM SHIKIMATE-5-P AND AMINO DONORS

The reaction mixtures contained 0.2 ml of extract, 5 μmoles of $MgCl_2$, 40 μmoles of Tris buffer, *p*H 8.2, 1 μmole of shikimate-5-P, and additions as indicated, in a total volume of 1 ml. Following incubation at 37° for 2 hours, aliquots were removed for the assay of anthranilate.

Additions	Yield of Anthranilate (μmole)
None	0
5.0 μmoles of L-aspartate	0.10
5.0 μmoles of L-glutamate	0.20
5.0 μmoles of L-glutamine	0.86
5.0 μmoles of L-asparagine	0.17
5.0 μmoles of NH_4Cl	0.18
5.0 μmoles of D-glutamine	0
20.0 μmoles of adenosine-phosphoramidate	0

L-Glutamine-(amide-N^{15}) was also suitably diluted and assayed for its N^{15} content.

RESULTS

Amino and Carbon Donors.—Of the various amino donors studied with shikimate-5-P, L-glutamine was the most effective (Table I). L-Aspartate, L-asparagine, L-glutamate, NH_4Cl, and adenosinephosphoramidate were poorly utilized.

With L-glutamine as the amino donor various carbon sources were tested for their capacity to substitute for shikimate-5-P (Table II). Shikimate was ineffective. The addition of shikimate and ATP, however, gave a conversion of 18% in contrast to the almost quantitative conversion of shikimate-5-P. This small yet significant conversion may be attributed to the presence of a kinase in these extracts capable of phosphorylating shikimate to shikimate-5-P. This seems to be reasonable in view of the recent demonstration of the existence of shikimate kinase in bacterial extracts (Fewster, 1962). Compound Z1, the 3-enolpyruvate ether of shikimate, and the two isomers of 6-amino-3,4,5-trihydroxycyclohexane carboxylate could not replace shikimate-5-P. Similarly, benzoate and *o*-hydroxybenzoate were not converted to anthranilate in the presence of L-glutamine.

Incorporation of Amide-N of Glutamine into Anthranilate.—Since glutamine contains two amino groups (*i.e.*, the α-amino and γ-amide-N), it was deemed necessary to determine the origin of the amino group of anthranilate. With this in view, a large scale isolation of anthranilate was carried out in the presence of glutamine-(amide-N^{15}), under conditions described in detail under Experimental. The results are presented in Table III. The close correlation between the atom per cent excess of the amide-N of glutamine and the isolated anthranilate demonstrates that the amino group of anthranilate arises from the amide-N of L-glutamine.

TABLE II

SYNTHESIS OF ANTHRANILATE FROM L-GLUTAMINE AND VARIOUS CARBON SOURCES

The reaction mixtures contained 0.2 ml of enzyme extract, 5 μmoles of $MgCl_2$, 40 μmoles of Tris buffer, *p*H 8.2, 5 μmoles of L-glutamine, and additions as indicated, in a total volume of 1.0 ml. Following incubation at 37° for 2 hours, aliquots were removed for the assay of anthranilate.

Additions	Yield of Anthranilate (μmole)
None	0
1.0 μmole shikimate	0
1.0 μmole shikimate + 1.0 μmole ATP	0.18
1.0 μmole shikimate-5-P	0.80
1.0 μmole of 3-enolpyruvylshikimate	0
1.0 μmole of 6-amino-3,4,5-trihydroxy-cyclohexanecarboxylate	0

TABLE III

INCORPORATION OF AMIDE-N OF GLUTAMINE INTO ANTHRANILATE

	Compound Analyzed	N^{15} (atom % excess)	Dilution Factor	N^{15} Corrected for Dilution (atom % excess)
1.	L-Glutamine-(amide-N^{15})	1.024	31.7	32.5
2.	Anthranilate	0.832	35.8	29.8

Cofactor Requirements.—Some of the enzyme extracts were incapable of synthesizing anthranilate from shikimate-5-P and L-glutamine unless they were supplemented with a pyridine nucleotide (NAD or NADP or their reduced equivalents). In order to establish the participation of a pyridine nucleotide, extracts were treated with charcoal to remove the bound pyridine nucleotides. These treated extracts were completely inactive. Addition of NAD, NADH, or NADP restored the synthesis to the normal values (Table IV). *Neurospora* nicotinamide adenine dinuclease (known to cleave only the oxidized form of the pyridine nucleotides) abolished the synthesis when added to nontreated extracts or to the reconstituted charcoal-treated extracts. Unlike NADP, which was required in substrate amounts for the complete synthesis, NAD was effective in catalytic amounts. The catalytic requirement for NAD can be explained as being due to the recycling of NADH to NAD by the highly active NADH oxidase normally present in these extracts. The restoration of synthesis with NADH can also be attributed to the NADH oxidase, for the simultaneous addition of *Neuropsora* nicotinamide adenine dinuclease to this system abolished the synthesis of anthranilate.

TABLE IV

REQUIREMENT FOR NAD IN THE CONVERSION OF SHIKIMATE-5-P + L-GLUTAMINE TO ANTHRANILATE

The incubation mixtures contained 0.2 ml of charcoal-treated extract (as described under Experimental), 1 μmole of shikimate-5-P, 5 μmoles each of L-glutamine and $MgCl_2$, 40 μmoles of Tris buffer, *p*H 8.2, and additions as indicated, in a total volume of 1.0 ml. The mixture was incubated at 37° for 2 hours.

Additions	Yield of Anthranilate (μmole)
None	0
0.08 μmole NAD	0.76
0.80 μmole NAD	0.76
0.80 μmole NAD + nicotinamide adenine dinuclease	0
0.08 μmole NADP	0.20
0.16 μmole NADP	0.35
0.80 μmole NADP	0.77
0.80 μmole NADP + nicotinamide adenine dinuclease	0
0.80 μmole NADH	0.76

TABLE V

STIMULATION OF ANTHRANILATE SYNTHESIS BY VARIOUS COMPOUNDS

The reaction mixture consisted of 0.5 μmole of shikimate-5-P, 2.5 μmoles of L-glutamine, 40 μmoles of Tris buffer *p*H 8.2, 2.0 μmoles of $MgCl_2$, 0.5 μmole of NAD, 1.0 μmole of the substances listed below, unless otherwise indicated, and 0.1 ml of crude *E. coli* B-37 extract of cells grown on yeast extract and casamino acids (2 mg of protein) in a final volume of 1 ml. The mixture was incubated at 37° for 2 hours.

Additions	Anthranilate Formed (μmole)
None	0.02
Boiled extract, 0.1 ml[a]	0.28
Glucose 6-phosphate	0.43
Ribose 5-phosphate	0.43
Fructose 6-phosphate	0.46
Fructose 1,6-diphosphate	0.48
Glyceraldehyde 3-phosphate	0.37
Lactate	0.18
Fumarate, 2.0 μmoles	0.38
NADH-regenerating system[b]	0.23
Phosphoenolpyruvate	0.01
Phosphoglyceric acid	0.05
ATP-regenerating system[c]	0.01
Pyruvate	0.05

[a] The boiled extract was prepared from extracts which did not require the addition of sugar phosphates for activity. [b] 0.15 mg alcohol dehydrogenase and 150 μmoles of ethanol. [c] 0.08 mg creatine phosphate transphosphorylase, 10 μmoles of creatine phosphate and 0.5 μmole of ATP.

Several compounds (Table V) were found to stimulate the synthesis of anthranilate from shikimate-5-P and L-glutamine. An *E. coli* B-37 extract prepared from cells grown on medium A supplemented with 0.2% casamino acids and 0.2% yeast extract, which did not synthesize anthranilate from shikimate-5-P, L-glutamine and NAD, best illustrates the stimulatory effect of glyceraldehyde 3-phosphate, ribose 5-phosphate, glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate, fumarate, or a NADH-regenerating system. In view of the effectiveness of the NADH-regenerating system in promoting the synthesis of anthranilate, the stimulation could be attributed to the regeneration of NADH since all of the compounds used could reduce NAD either directly or indirectly. That neither phosphoenolpyruvate nor phosphoglycerate stimulated the synthesis of anthranilate is significant in view of the further studies reported in the following paper (Rivera and Srinivasan, 1963).

TABLE VI

METAL REQUIREMENTS FOR THE SYNTHESIS OF ANTHRANILATE

The reaction mixture consisted of 0.5 μmole of shikimate-5-P, 2.5 μmoles of L-glutamine, 50 μmoles of Tris buffer, *p*H 8.2, 5 μmoles of the inorganic compounds indicated below, 0.5 μmole of NAD, 0.1 ml of dialyzed crude *E. coli* B-37 extract, 0.1 ml of Dowex-50-treated boiled extract, in a final volume of 1 ml. The reaction mixture was incubated at 37° for 2 hours.

Additions	Anthranilate Formed (μmole)
None	0
Mg^{++}	0.16
Mn^{++}	0.09
Fe^{++}	0.18
Mg^{++}, Mn^{++}	0.10
Mg^{++}, Zn^{++}	0
Mg^{++}, Co^{++}	0
Mg^{++}, Fe^{+++}	0.08
Mg^{++}, Fe^{++}	0.13
Mg^{++}, Ca^{++}	0.11
Mg^{++}, Na^{+}	0.16
Mg^{++}, Al^{+++}	0
Mg^{++}, Cu^{++}	0
Mg^{++}, Cd^{++}	0

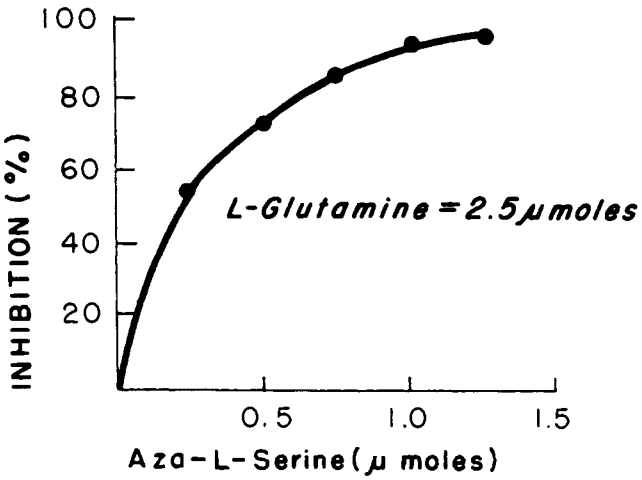

FIG. 1.—Effect of aza-L-serine on the formation of anthranilate. In addition to aza-L-serine, the incubation mixtures contained 0.5 μmole of shikimate-5-P, 2.5 μmoles of L-glutamine, 2 μmoles of $MgCl_2$, 20 μmoles of Tris buffer, *p*H 8.2 and 0.2 ml of enzyme extract in a total volume of 0.5 ml. The incubation and assay procedures were as in Table I.

The metal requirement of the enzyme system was investigated by using a cell-free extract dialyzed against potassium phosphate buffer (0.033 M, *p*H 7.4). A boiled extract prepared from the undialyzed extract and treated with Dowex-50 Na^+ form was included in the reaction mixture to furnish the necessary cofactors. In the absence of added cations there was no synthesis of anthranilate from shikimate-5-P and L-glutamine (Table VI). Mg^{++}, Mn^{++}, or Fe^{++} promoted the synthesis, Mn^{++} being far less effective than Mg^{++} or Fe^{++}. While Al^{+++}, Cd^{++}, Cu^{++}, Co^{++}, and Zn^{++} completely inhibited the reaction in the presence of Mg^{++}, Fe^{+++} and Ca^{++} decreased the activity only partially.

Inhibition.—Fluoride (5 μmoles/ml) inhibited the

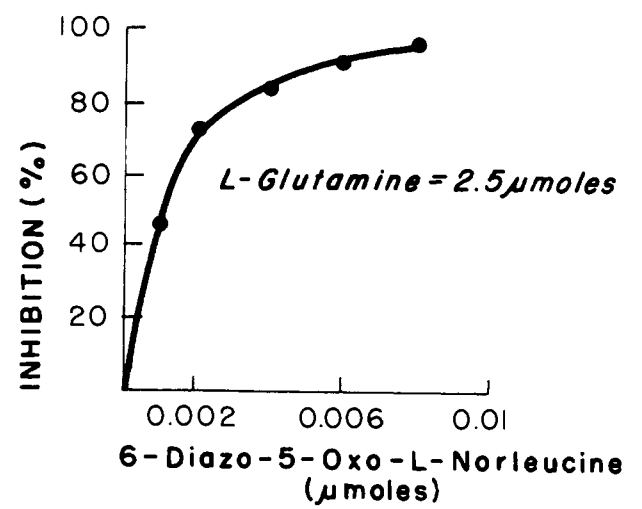

FIG. 2.—Effect of 6-diazo-5-oxo-L-norleucine on the formation of anthranilate. The procedure was the same as in Figure 1 except for the substitution of 6-diazo-5-oxo-L-norleucine instead of aza-L-serine.

reaction completely. Low concentrations of phosphate were without effect, but levels of 3×10^{-2} M phosphate reduced the synthesis by 50%. Levenberg *et al.* (1957) have shown that aza-L-serine and 6-diazo-5-oxo-L-norleucine, as structural analogs of glutamine, strongly inhibit the conversion of formylglycinamide ribotide to formylglycinamidine ribotide. Accordingly, several glutamine analogs were studied to determine their effect upon the synthesis of anthranilate from shikimate-5-P and L-glutamine. The inhibition of synthesis by aza-L-serine and DON are illustrated in Figures 1 and 2. At a concentration of 5.0×10^{-4} M aza-L-serine (glutamine concentrations 5.0×10^{-3} M) reduced the synthesis by 50%. On the other hand, DON inhibited the synthesis more markedly: concentrations as low as 2.0×10^{-6} M resulted in a 50% inhibition. Thus, the latter was at least 200 times more effective than aza-L-serine in reducing the synthesis of anthranilate by 50%. The results were not influenced by preincubation with the inhibitors. Even at similar concentrations other analogs such as γ-glutamylhydrazine, *O*-carbazyl-DL-serine, and diazoacetic ester of 6-hydroxynorleucine did not affect the system to any appreciable extent.

Discussion

Of the various carbon and amino donors tried only shikimate-5-P and L-glutamine were capable of being converted to anthranilate. Z1, the 3-enolpyruvyl ether of shikimate, was neither a substrate nor an inhibitor for the formation of anthranilate from shikimate-5-P and L-glutamine. The availability of mutants (Davis and Mingioli, 1953) accumulating Z1 in the culture medium and requiring the quintuple supplementation with phenylalanine, tyrosine, tryptophan, *p*-aminobenzoate, and *p*-hydroxybenzoate for growth would suggest that Z1 or a derivative thereof is probably an intermediate after shikimate-5-P. This problem is examined in the following paper.

The effective inhibition of the anthranilate-forming system by the structural analogs of L-glutamine is extremely interesting in view of the sparing effect of the aromatic amino acids, phenylalanine, tyrosine, and tryptophan on aza-L-serine inhibition in whole cells of *E. coli* (Kaplan *et al.*, 1959). However, the recent studies of Brock and Brock (1961) suggest that the sparing action may be explained on the basis of the aromatic amino acids preventing the entry of aza-L-serine into the cells.

Of the two analogs of glutamine, DON and aza-L-serine, the former was the more potent inhibitor in the present studies. In the conversion of formylglycinamide ribotide to the formylglycinamidine ribotide, an enzymatic reaction involving glutamine, DON was only forty times more effective than aza-L-serine, whereas in the anthranilate system DON was at least 200 times more effective. We have recently demonstrated that the amino group of *p*-aminobenzoate also stems from the amide-N of glutamine (Weiss and Srinivasan, 1959). However, in this reaction DON was only forty times more inhibitory than aza-L-serine. Indeed, when the anthranilate and *p*-aminobenzoate-forming systems compete for glutamine, DON inhibits the more sensitive anthranilate system effectively at low concentrations (Srinivasan and Weiss, 1961).

The conversion of shikimate-5-P and L-glutamine required the participation of a pyridine nucleotide. However, certain extracts were found to be inactive unless they were supplemented with a sugar phosphate (*i.e.*, glucose-6-P, ribose-5-P, and the like) or with a reducing system capable of regenerating NADH. The absence of such a requirement with some extracts could be attributed to the presence of endogenous substrates capable of reducing NAD to NADH.

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