*THE BIOSYNTHESIS OF p-AMINOBENZOIC ACID**

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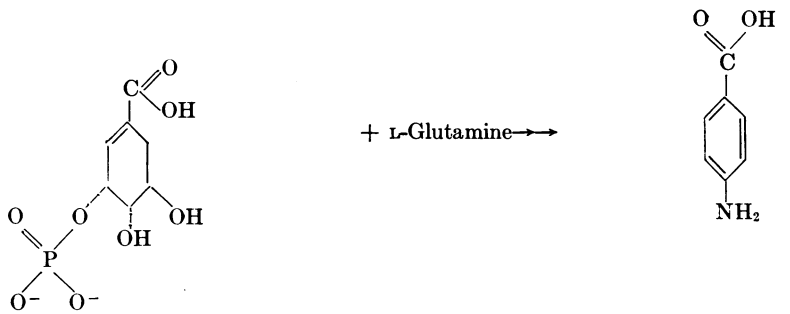
Nutritional studies with mutants of *Escherichia coli*[1] and *Neurospora*[2] have established shikimic acid as an intermediate in the biosynthesis of phenylalanine, tyrosine, tryptophan, and *p*-aminobenzoic acid. Subsequent investigations have revealed other mutants which accumulate shikimic acid-5-phosphate (I) or "Z1" in their medium and require all the above compounds for growth.[3] Compound "Z1" (probably the 5-enolpyruvate of shikimic acid[4]) originates at a later stage in the biosynthetic sequence by a condensation of shikimic-5-P and phosphoenolpyruvate.[5] These observations suggest that "Z1" may be the branching point in the formation of these aromatic compounds in nature. However, recent work with enzyme extracts of *E. coli* mutants have shown that anthranilic acid (the benzenoid intermediate in the biosynthesis of tryptophan) is formed from shikimic-5-P and L-glutamine.[6] This finding has led us to study the biosynthesis of *p*-aminobenzoic acid as well. We demonstrate in the present paper that *p*-aminobenzoic acid is formed in cell-free extracts of yeast from shikimic-5-P and L-glutamine.



(I)

Material and Methods.—Shikimic acid-5-phosphate was isolated from the culture medium of *Aerobacter aerogenes* strain A 170–40 as the barium salt[7] and converted to the potassium salt for the enzymatic experiments. Yeast concentrate and liver concentrate were obtained from Sigma Chemical Co., St. Louis.

Preparation of enzyme extract: 50 g of baker's yeast were disrupted by freezing and thawing three times with the aid of liquid nitrogen. The viscous material was stirred with 50 ml of $M/10$ potassium phosphate buffer (pH 7.4) for 4 hours at 2°, centrifuged at 15,000 g for 20 minutes and the cell-free extract was extensively dialyzed against $M/30$ potassium phosphate buffer (pH 7.4; 6 changes, 3 liters each time). The dialyzed extract was used in the experiments described below.

Enzymatic system and assay of p-aminobenzoic acid: The incubation mixtures contained 0.4 ml of extract (4.8 mg of protein), 2 μmoles of $MgCl_2$, 1 mg of yeast concentrate, 50 μmoles of Tris buffer (pH 7.4), shikimic-5-P or glutamine, plus additions in a total volume of 1 ml. After incubation at 30°, samples were removed at 4, 8, and 24 hours, brought to pH 1.0 with 6 N HCl, and used for the microbiological determination of PABA with a double auxotroph of *E. coli* (107–14) requir-

ing histidine and PABA.[8] The assay medium (final volume 10 ml) was prepared by supplementing mineral medium A[9] with 75 μg of L-histidine per ml.

Conversion of uniformly labeled C^{14}-shikimic-5-P to p-aminobenzoic acid:[10] 312 μmoles of C^{14}-shikimic-5-P, 1560 μmoles of L-glutamine, 625 mg of yeast concentrate, 3125 μmoles of tris buffer (pH 7.4), 1250 μmoles of $MgCl_2$ and 250 ml enzyme extract in a total volume of 650 ml, were incubated for 24 hours at 30°. After incubation, the mixture was placed in a boiling water bath for 10 min, cooled, and the pH adjusted to 3.0 with 4 ml of 6 *N* H_2SO_4. The precipitated protein was removed by centrifugation, and aliquots of the supernatant solution were analyzed for PABA at three levels in duplicate. To 625 ml of this supernatant solution, which contained 2.29 mg of PABA, 100 mg of unlabeled PABA were added. The PABA was then isolated by continuous ether extraction and was recrystallized from H_2O to constant radioactivity and melting point.

Results and Discussion.—Amino and carbon donors: Of the various amino donors tried with shikimic-5-P, glutamine was the most effective (Table 1). Aspartic acid, asparagine, and glutamic acid were poorly utilized. Ammonium chloride was unable to replace glutamine as the amino donor at either pH 7.4 or 8.2.

TABLE 1

SYNTHESIS OF *p*-AMINOBENZOIC ACID FROM SHIKIMIC-5-P AND AMINO DONORS*

Substrates and Additions	Yield of *p*-Aminobenzoic Acid (μg) 4 Hours	8 Hours	24 Hours
0.5 μmole of shikimic-5-P	0	0	0
+ L-glutamine	2.4	4.1	4.70
+ L-glutamic acid	...	...	0.06
+ L-asparagine	...	...	0.09
+ L-aspartic acid	...	...	0.11
+ NH_4Cl (pH 7.4)	...	...	0.05
+ NH_4Cl (pH 8.2)	...	...	0.05

* 2.5 μmoles of each amino donor were used in these experiments.

With glutamine as the amino donor, various carbon sources were studied for their capacity to substitute for shikimic-5-P (Table 2). Shikimic acid alone was poorly

TABLE 2

SYNTHESIS OF *p*-AMINOBENZOIC ACID FROM L-GLUTAMINE AND VARIOUS CARBON SOURCES

Substrates and Additions	Yield of *p*-Aminobenzoic Acid (μg) 4 Hours	8 Hours	24 Hours
2.5 μmoles of glutamine	0.88	0.80	0.88
+ 0.1 μmole of shikimic-5-P	1.90	2.50	2.60
+ 0.5 μmole of shikimic-5-P	2.40	3.70	4.80
+ 0.5 μmole of shikimic acid	1.30	1.40	1.50
+ 0.5 μmole of shikimic acid + 0.5 μmole of ATP	2.50	2.50	3.00
+ 0.5 μmole of Z1	0.09	0.07	0.06

utilized and addition of ATP enhanced the synthesis to half the value obtained using shikimic-5-P as the carbon source. "Z1" was not only inactive but reduced the blank value considerably, suggesting an inhibitory effect on the synthesis. Irrespective of the amino donor employed, *p*-hydroxybenzoic acid was not converted to PABA. This confirms the earlier observations of Professor B. D. Davis derived from a study of the growth requirements of *E. coli* aromatic auxotrophs.[11] These results demonstrate that shikimic-5-P is the carbon donor in the formation of PABA. The increased synthesis obtained by the addition of ATP to shikimic acid indicates

the presence of a kinase in these extracts for the phosphorylation of shikimic acid to shikimic-5-P.

Glutamine alone added to the extracts without shikimic-5-P resulted in a significant synthesis, indicating the presence of possible precursors in the yeast extract itself. The extracts were therefore assayed for shikimic acid and shikimic-5-P.[12] A typical extract, with a synthesis of 0.9 μg of PABA from glutamine alone, was found to contain 11 μg of shikimic acid and 22.5 μg of shikimic-5-P per ml. The presence of these amounts of substrate could satisfactorily explain the formation of PABA from glutamine in the absence of any extraneous addition of shikimic-5-P. It was further observed that extracts with low blank values could not be stored in the deep freeze even for a few days without losing considerable activity suggesting that the enzyme or enzymes responsible for the conversion of shikimic-5-P and L-glutamine to PABA are protected by their own substrate. The apparent presence of such large amounts of substrate even after extensive dialysis is of interest. It simulates the well-known tight binding of coenzymes in other enzyme systems.

Since the amount of PABA formed was small, it was felt desirable to establish, unequivocally, the nature of the end product. With this view in mind, uniformly labeled C^{14}-shikimic-5-P and L-glutamine were incubated with enzyme extracts, under conditions described earlier in detail. The molar specific activity of the isolated *p*-aminobenzoic acid compared favorably with the molar specific activity of shikimic-5-P, suggesting a 1:1 conversion (Table 3). This experiment lends

TABLE 3

CONVERSION OF SHIKIMIC ACID-5-PHOSPHATE-C^{14} TO *p*-AMINOBENZOIC ACID

Compound	Activity of Carbon* cpm
Shikimic acid-5-phosphate (barium salt)	127×10^3
p-Aminobenzoic acid†	133×10^3

* Counts per minute at "infinite" thickness under standard conditions divided by the fraction of carbon in the compound.
† Corrected for dilution.

further evidence to the identity of the enzymatic product as PABA.

Nature of co-factors: The dialyzed yeast extracts were completely inactive unless they were fortified with yeast concentrate or liver concentrate (Table 4). The

TABLE 4

CO-FACTOR REQUIREMENTS FOR THE SYNTHESIS OF *p*-AMINOBENZOIC ACID

Additions	Yield of *p*-Aminobenzoic Acid (μg) 4 Hours	8 Hours	24 Hours
None	0.11	0.10	0.21
Yeast concentrate (1 mg)	2.80	3.20	4.00
Liver concentrate (1 mg)	2.50	2.60	2.70
Boiled extract of *E. coli* B_{37}*	2.20	2.60	3.00
Yeast concentrate (1 mg) + DPNase	1.36	1.34	1.50

The incubation mixture contained 0.5 μmole of shikimic-5-P; 2.5 μmoles of L-glutamine; 2 μmoles of $MgCl_2$; 50 μmoles of Tris buffer, pH 7.4; 0.4 ml of enzyme extract plus additions in a total volume of 1 ml.
* Kindly furnished by Professor J. S. Gots.

yeast and liver concentrates employed in these experiments contain a mixture of nucleoside monophosphates, diphosphates, DPN, TPN, CoA, and possibly trace amounts of nucleoside triphosphates. *Neurospora* DPNase[13] decreased the synthesis of PABA, in the presence of yeast concentrate, by 50–60 per cent. How-

ever, neither DPN nor DPN + ATP were able to replace the yeast concentrate. Thus, the exact nature of the co-factors involved is obscure, and studies are in progress toward its elucidation.

Since anthranilic acid and *p*-aminobenzoic acid are both derived from shikimic-5-P and L-glutamine, it would be of interest to determine whether a common intermediate may not be involved in the biosynthesis of these two compounds.

Summary.—With cell-free extracts of baker's yeast, it has been demonstrated that *p*-aminobenzoic acid is formed from shikimic acid-5-phosphate and L-glutamine. The co-factor requirements of the system are also briefly discussed.

We are extremely grateful to Professor B. D. Davis for providing us with the mutants used in this work.

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The following abbreviations have been used: shikimic-5-P, shikimic acid-5-phosphate; PABA, *p*-aminobenzoic acid; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; ATP, adenosinetriphosphate; CoA, Coenzyme A; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

[1] Davis, B. D., *J. Biol. Chem.*, **191**, 315 (1951); *Advances in Enzymology*, **16**, 287 (1955).

[2] Tatum, E. L., in *Plant Growth Substances*, ed. F. S. Koog (Madison: University of Wisconsin Press, 1951).

[3] Davis, B. D., and E. S. Mingioli, *J. Bact.*, **66**, 129 (1953).

[4] Gilvarg, C., and B. D. Davis, unpublished observations; Davis, B. D., *Arch. Biochem. Biophys.*, **78**, 497 (1958).

[5] Kalan, E. B., and B. D. Davis, unpublished observations.

[6] Srinivasan, P. R., *J. Am. Chem. Soc.*, **81**, 1772 (1959).

[7] Weiss, U., and E. S. Mingioli, *J. Am. Chem. Soc.*, **78**, 2894 (1956).

[8] We are indebted to Dr. E. Borek for providing us with this mutant, which was originally isolated by Dr. B. D. Davis.

[9] Davis, B. D., and E. S. Mingioli, *J. Bact.*, **60**, 17 (1950).

[10] C^{14}-shikimic-5-P was isolated from the culture medium of *Aerobacter aerogenes* strain A 170-40 grown in the presence of uniformly labeled glucose-C^{14}.

[11] Davis, B. D., *J. Bact.*, **64**, 729 (1952).

[12] Shikimic acid was estimated microbiologically (Davis, B. D. and U. Weiss, *Arch., Exp. Path. U. Pharmakol.*, **220**, 1, 1953) and shikimic-5-P was determined by assaying the skimic acid released after cleavage with acid phosphatase.

[13] *Neurospora* DPNase was a generous gift of Professor N. O. Kaplan.

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*GLUCOSE INHIBITION AND THE DIAUXIE PHENOMENON**

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Salmonella typhimurium, strain LT2, is able to utilize glucose, glycerol, pyruvate, and Krebs cycle compounds such as citrate, succinate, fumarate, and malate as sole sources of carbon and energy for growth. Spontaneous mutants (C^-) have been isolated from this strain which have retained the wild type ability to utilize the Krebs cycle compounds but are unable to utilize glucose or other carbohydrates, glycerol, or pyruvate as sole carbon and energy sources.[1] Although growth factor