THE FORMATION OF 3-ENOLPYRUVYL SHIKIMATE 5-PHOSPHATE IN EXTRACTS OF ESCHERICHIA COLI*

Judith G. Levin and David B. Sprinson+

Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, N.Y.

Received July 29, 1960

Compound Z1, an acid-labile and nutrionally inactive derivative of shikimic acid, is excreted by several aromatic auxotrophs blocked beyond shikimate (1). Since mutants which accumulate large amounts of Z1 require the full aromatic supplement of phenylalanine, tyrosine, tryptophan, p-aminobenzoic acid and p-hydroxybenzoic acid, it was suggested that Z1 is a common intermediate between shikimate and the aromatic compounds(2). A non-crystalline barium salt of Z1 was isolated from culture filtrates, and on the basis of its hydrolysis by dilute acid to one mole each of pyruvic and shikimic acids and its consumption of one mole of periodate, Z1 was assigned the structure of the 3- or 5-enolpyruvate ether of shikimic acid (3). The function of Z1 as an intermediate in aromatic biosynthesis, and its structure as the 5-enolpyruvate ether of shikimic acid, were rationalized by a suggestion, first made several years ago, that such a structure might undergo a Claisen rearrangement and dehydration to yield prephenic acid (4).

A compound with the chromatographic and chemical properties of Z1 was shown to be formed in cell-free extracts of E. coli from shikimate 5-phosphate and enolpyruvate phosphate (5). The present report is concerned with a further study of this reaction. The results indicate that the first product formed is 3-enolpyruvyl shikimate 5-phosphate which is then dephosphorylated to Z1, and that Z1 is 3- rather than 5-enolpyruvyl shikimate.

* This work was supported by grants from the American Cancer Society, the American Heart Association, and the National Institutes of Health of the United States Public Health Service.

+ Career Investigator of the American Heart Association.

Experimental

Preparation of Extract - E. coli K-12 mutant 58-278, a phenylalanine auxotroph (6) which accumulates prephenic acid (7), was grown with aeration for 18 hours at 35° on Medium A (1) enriched with 0.2 % yeast extract and 0.2 % Casamino Acids. The cells were harvested by centrifugation at 3°, washed two times with 1/30 M phosphate buffer, pH 7.2, suspended in 0.01 M Tris (tris (hydroxymethyl) amino methane) buffer, pH 8.2 (20 ml per 5 gm of wet bacteria), and subjected to sonic oscillation at 9 kc, with cooling, for 30 minutes. All subsequent operations were carried out at 2°. The clear supernatant solution, obtained by centrifugation, was treated with 0.5 ml of 2 % protamine sulfate solution per 70 mg of protein (determined spectrophotometrically (8)), and the precipitate was removed by centrifugation and discarded. The supernatant solution was treated with solid $(NH_4)_2SO_4$ in order to obtain a fraction precipitating between 0.4 and 0.6 saturated $(NH_4)_2SO_4$. The precipitate was dissolved in 0.01 M Tris buffer and was dialyzed extensively against this buffer.

Incubation Procedures and Determination of Constituents - The reaction mixture contained 1 μmole of shikimate 5-phosphate, 1 μmole of enolpyruvate phosphate, 50 μmoles of Tris buffer, pH 8.2, 10 μmoles of KF (where added), and 0.05 ml of extract (1 mg of protein), in a final volume of 1.4 ml; it was incubated at 37° for 18 hours. Proteins were precipitated by the addition of 0.8 ml of 25 % trichloroacetic acid to 1.2 ml of incubation mixture, and removed by centrifugation and filtration. For the determination of Z1 a 0.2 ml aliquot of the filtrate was heated in a boiling water bath for 10 minutes, and used for the assay of shikimate (9). A 0.8 ml aliquot was used for phosphate determination (10). In the experiments described below Z1 was determined as follows: an aliquot of the incubation mixture was acidified with 6 N HCl to a final concentration of 0.1 N, proteins were removed by centrifugation, and the supernatant solution was treated as described above.

To determine pyruvate released by acid hydrolysis, the unreacted enolpyruvate phosphate in the incubation mixture was converted to lactate enzymatically (11), 6 N HCl was added to a final concentration of 0.1 N, and

proteins were removed by centrifugation. The supernatant solution was heated in a boiling water bath for 10 minutes, and used for the determination of pyruvate (12).

To estimate unreacted shikimate 5-phosphate and 3-enolpyruvyl shikimate 5-phosphate, 0.8 ml of the reaction mixture was further incubated for 1 hour at 37° with 50 μmoles of Tris buffer, pH 8.2, 10 μmoles of $MgCl_2$, and 50 μg of intestinal alkaline phosphatase (Worthington), in a final volume of 1.2 ml. An aliquot was used directly for shikimate assay. Another aliquot was acidified with 6 N HCl to give a concentration of 0.1 N, heated in a boiling water bath for 10 minutes, and assayed for additional shikimate produced.

Table I

Effect of Fluoride on Formation of Z1 and Release of Orthophosphate

The reaction mixture contained 1 μmole of shikimate 5-phosphate and 1 μmole of enolpyruvate phosphate; it was incubated and analyzed as described under "Experimental".

Fluoride addition	Compound Z1 formed	Orthophosphate released and calculated: Found	Calculated from Z1 formed	Calculated from Z1 + ESP* formed
	μmoles	μmoles	μmoles	μmoles
No F^-	0.45	1.06	0.90	
With F^-	0.10	0.68	0.20	0.67†

*ESP, 3-enolpyruvyl shikimate 5-phosphate.
†See Table II, and "Discussion".

Results

It may be seen from Table I that incubation of the bacterial extract with equimolar amounts of shikimate 5-phosphate and enolpyruvate phosphate resulted in the formation of a 45 % yield of Z1. The inorganic phosphate released was always slightly, but significantly, higher than that calculated for the reaction: shikimate 5-phosphate + enolpyruvate phosphate ⟶ Z1 + 2 orthophosphate. In the presence of fluoride, the formation of Z1 was strongly inhibited, but the release of orthophosphate was much higher than that calculated

Table II

Evidence for the Formation of 3-Enolpyruvyl Shikimate 5-Phosphate

The reaction mixture contained 1 μmole of shikimate 5-phosphate, 1 μmole of enolpyruvate phosphate and 10 μmoles of KF; it was incubated and analyzed as described under "Experimental".

Compound determined	Found after acid hydrolysis	Calculated from Z1 formed	Released by alkaline phosphatase*	Released by alkaline phosphatase*, followed by acid hydrolysis
	μmoles	μmoles	μmoles	μmoles
Pyruvate	0.42	0.10		
Shikimate	0.10+		0.26	0.47

* Control experiments, with shikimate 5-phosphate as a substrate, showed that in the presence of the fluoride used in these incubations the alkaline phosphatase was somewhat inhibited. The shikimate recovered in all its derivatives is therefore less than 1 μmole.
+ This value is designated "Z1" in Table I.

from this equation.

Further analysis of the incubation mixtures containing fluoride is presented in Table II. It may be seen that much more pyruvate was liberated by hydrolysis with dilute acid than would be expected from the amount of Z1 formed, and that only 0.26 μmole of shikimate was released by alkaline phosphatase from unreacted shikimate 5-phosphate, whereas approximately 0.90 μmole should have remained unreacted if the 0.10 μmole of Z1 were the only compound formed. Furthermore, when these incubation mixtures were subjected to acid hydrolysis after treatment with alkaline phosphatase, an additional 0.47 μmole of shikimate was set free. This must have come from a compound, which after dephosphorylation, resembled Z1 in giving rise to shikimic acid on hydrolysis with dilute acid.

Discussion

It would appear from these results that shikimate 5-phosphate and enolpyruvate phosphate are converted (in the presence of fluoride) to a compound which, like Z1, yields pyruvate on hydrolysis with dilute acid, but unlike Z1, does not give rise to shikimic acid on acid hydrolysis until after dephosphorylatio by the addition of phosphatase. On the reasonable assumption that the phosphate

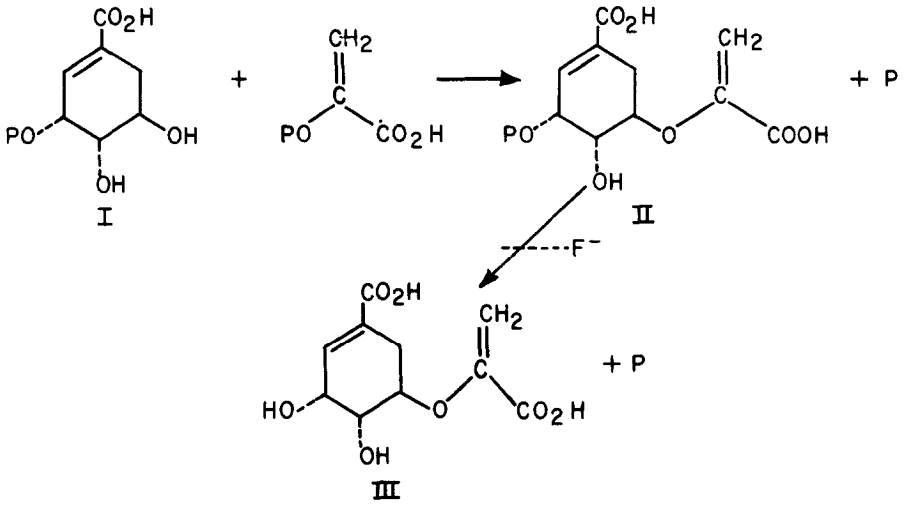

Fig. 1

in this compound is still attached to the hydroxyl group on C5 and that dephosphorylation produces Z1, it may be suggested that shikimate 5-phosphate (I) and enolpyruvate phosphate react to form 3-enolpyruvyl shikimate 5-phosphate (II) and orthophosphate (Fig. 1). In extracts without fluoride, and presumably in intact cells of appropriate mutant strains, 3-enolpyruvyl shikimate 5-phosphate is dephosphorylated to Z1 (III). In extracts with fluoride, dephosphorylation is inhibited and the phosphorylated compound is accumulated. These results are in accord with more recent structural studies on Z1 which indicate that it is 3-enolpyruvyl shikimate (13).

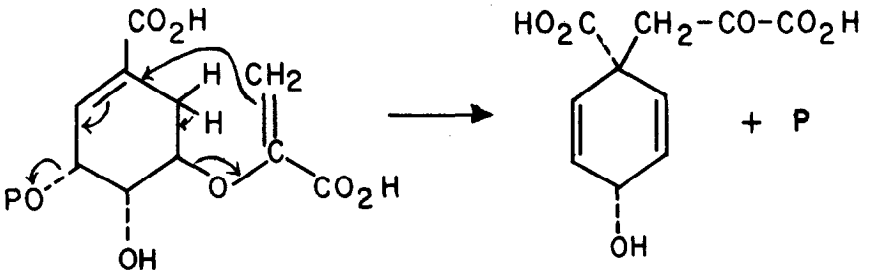

Fig. 2

The phosphate released in excess of that calculated from the Z1 produced (Table I) may be explained by assuming the formation of 3-enolpyruvyl shikimate 5-phosphate in addition to Z1. In the presence of fluoride, 0.68 μmole of phosphate is liberated from 1 μmole of shikimate 5-phosphate and 1 μmole of enolpyruvate phosphate; this corresponds to 0.2 μmole calculated from the formation of 0.1 μmole of Z1, plus the 0.47 μmole expected from the formation of 0.47 μmole of the postulated 3-enolpyruvyl shikimate 5-phosphate (Table II). Without fluoride this compound is largely dephosphorylated to Z1 but, as pointed out earlier, the amount of inorganic phosphate released is somewhat higher than that expected from the yield of Z1 alone. Results obtained by the analysis of incubation mixtures without fluoride were in accord with the view that they contained small amounts of 3-enolpyruvyl shikimate 5-phosphate.

Attempts to demonstrate the conversion of Z1 to prephenate in extracts of *E. coli* (K-12 mutant 58-278, or wild-type) were unsuccessful, and it was shown that Z1 remained unchanged under all conditions tried (14). However, the formation of prephenate from shikimate 5-phosphate and enolpyruvate phosphate could be readily demonstrated (15). These observations suggest that 3-enolpyruvyl shikimate 5-phosphate, rather than Z1, is the active intermediate in prephenate formation. A mechanism for this conversion is suggested in Fig. 2. This mechanism implies that C1 of prephenate has the stereochemistry shown.

Further work is in progress to isolate 3-enolpyruvyl shikimate 5-phosphate and to study its conversion to prephenate.

We are greatly indebted to Professor B. D. Davis for generous gifts of shikimate 5-phosphate and Z1, and for the mutant strains used in this work.

References

1. B. D. Davis and E. S. Mingioli, J. Bacteriol., 66, 129 (1953).
2. B. D. Davis, Advances in Enzymol., 16, 247 (1955).
3. C. Gilvarg, Private communication.
4. R. B. Woodward, Private communication.

5. E. B. Kalan and B. D. Davis, Private communication.

6. S. Simmonds, J. Biol. Chem., 185, 755 (1950).

7. M. Katagiri and R. Sato, Science, 118, 250 (1953);
B. D. Davis, ibid., 118, 251 (1953).

8. O. Warburg and W. Christian, Biochem. Z., 310, 384 (1941-42).

9. B. D. Davis and U. Weiss, Arch. exp. Path. u. Pharmakol., 220, 1 (1953).

10. J. H. Bruemmer and B. L. O'Dell, J. Biol. Chem., 219, 283 (1956).

11. A. Kornberg and W. E. Pricer, Jr., J. Biol. Chem., 193, 481 (1951).

12. T. E. Friedemann and G. E. Haugen, J. Biol. Chem., 147, 415 (1943).

13. I. J. Borowitz and D. B. Sprinson, Unpublished observations.

14. J. G. Levin and D. B. Sprinson, Unpublished observations.

15. M. J. Clark and D. B. Sprinson, Unpublished observations.