Studies of the Mechanism of Anthranilate Synthase Reaction*

H. Tamir and P. R. Srinivasan

DEPARTMENT OF BIOCHEMISTRY, COLLEGE OF PHYSICIANS AND SURGEONS, COLUMBIA UNIVERSITY, NEW YORK, NEW YORK

Communicated by David Rittenberg, February 4, 1970†

Abstract. The enzyme anthranilate synthase catalyzes the formation of anthranilate from either chorismate and glutamine or chorismate and ammonia. In the aromatization of chorismate, a hydroxyl group and an enolpyruvyl group must be eliminated. Elimination of the enolpyruvyl group of chorismate is accompanied by protonation to form pyruvate. The source of this proton was investigated by performing the enzymatic reaction in 99.7 per cent D₂O.

The isolated pyruvate contained close to an atom of deuterium in the methyl group. High resolution mass spectra also revealed that about 6 per cent of the deuterio pyruvate contains a -CHD₂ species. Thus, the results obtained conclusively demonstrate that in the formation of the pyruvate, the third hydrogen of the methyl group arises from water and not by intramolecular shift of a hydrogen from the ring of chorismate.

Anthranilate synthase catalyzes the formation of anthranilate from either chorismate and glutamine or chorismate and ammonia (Fig. 1). In Enterobacteriaceae, the enzyme exists as an aggregate with the next enzyme in the tryptophan pathway, anthranilate-5-phosphoribosyl-pyrophosphate phosphoribosyl-transferase.¹ The aggregate is capable of utilizing either glutamine or ammonia as amino donor. The anthranilate synthase protein devoid of the transferase, termed component I, can utilize only ammonia as amino donor.

Earlier chemical studies from our laboratories have established that amination by the amide nitrogen of glutamine occurs at carbon atom 2 of chorismate.² In

\[
\text{Fig. 1.—Postulated scheme for the anthranilate synthase reaction.}
\]

547
the aromatization of chorismate, a hydroxyl group and an enolpyruvyl group must be eliminated. Elimination of the enolpyruvyl group of chorismate is accompanied by protonation to form pyruvate. The source of this proton has been investigated by Zalkin and Kling who performed the enzymatic reaction in tritiated water. These authors concluded that the third methyl proton originates from chorismate and not from the water. Recently, Onderka and Floss have reported that tritium from chorismate-2-T was not incorporated into the leaving enolpyruvyl group, yet tritium from tritiated water was incorporated to some degree in the pyruvate but not in the anthranilate. Since the above conflicting results could be reconciled by invoking isotope effects, it was of interest to study the mechanism of formation of pyruvate in 99.7 % D₂O with the homogenous anthranilate synthase isolated in our laboratory. The possibility of non-enzymatic exchange was minimized by trapping the pyruvate formed with p-bromophenylhydrazine. The results reported here show that the third proton of the pyruvate originates from water.

**Material and Methods.** The purification of the anthranilate synthase has been described. Just prior to use, the enzyme (spec. act., 43) was dialyzed for 2 hr against D₂O containing: 0.1 M Tris-chloride buffer, pH 7.5, 10⁻⁴ M EDTA, 10⁻⁴ M dithiothreitol, and 10⁻⁴ M MgCl₂. The incubation mixture contained in a total volume of 25 ml of D₂O; 200 μmoles of chorismic acid, 500 μmoles of glutamine, 125 μmoles of MgCl₂, 1.25 mmoles of Trischloride buffer, pH 7.5, 300 μmoles of p-bromophenylhydrazine, and 200 units of the enzyme dialyzed against D₂O. The Tris-buffer was previously lyophilized and dissolved in D₂O. The other reactants were directly dissolved in D₂O. Such a procedure does not appreciably alter the concentration of D₂O. The reaction occurred at 37°C for 4 hr and its course was followed by assaying the amount of glutamate formed. Under these conditions a conversion of over 70% was achieved. The control incubation mixture contained 100 μmoles of sodium pyruvate and all the other components except glutamine and chorismate.

At the end of the incubation, the mixture was acidified to pH 5 and allowed to stand overnight at 4°C. The precipitated hydrazone was collected by centrifugation, washed with H₂O, and crystallized from ethanol:H₂O. In those experiments where anthranilate was isolated, the mother liquor was further acidified to pH 3 and extracted with ether. The ether was removed and the crude anthranilic acid was recrystallized from hot water and subjected to further purification by sublimation in vacuo.

The deuterium content of the various compounds were analyzed by the method of Ponticorvo and Rittenberg. A high resolution mass spectrometer CEC 21-110 was used for investigating the mass spectra of the phenylhydrazones. The samples were analyzed at 70 ev and the block temperature was 115°C.

**Results and Discussion.** The results presented in Table 1 establish that close to one atom of deuterium is incorporated into pyruvate when the enzymatic reaction occurs in D₂O. In the control experiment in which pyruvate is incubated with the enzyme, negligible incorporation of deuterium from D₂O into the

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Pyruvate formed † (μmoles)</th>
<th>Pyruvate p-bromo-phenylhydrazine*</th>
<th>Anthranilic acid*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>181</td>
<td>86</td>
<td>1.7</td>
</tr>
<tr>
<td>2</td>
<td>170</td>
<td>89</td>
<td>...</td>
</tr>
<tr>
<td>Control</td>
<td>...</td>
<td>2.1</td>
<td>...</td>
</tr>
</tbody>
</table>

* The values of percentages of excess deuterium were calculated for one position.
† Since the stoichiometry of the reaction catalyzed by our enzyme preparation is 1:1, the assay of glutamate gives a measure of the pyruvate formed.
phenylhydrazone occurs. The other product of the enzymatic reaction, anthranilic acid, also contained very little deuterium.

The above results were confirmed and extended by analyzing the mass spectra of the \( p \)-bromophenylhydrazones. The location of the deuterium as well as its amount can be determined easily by comparing the mass spectra of the chemically prepared \( p \)-bromophenylhydrazone with the \( p \)-bromophenylhydrazone isolated from the enzymatic reaction mixture. The exact mass of the ions was determined using perfluorokerosene (Penninsular Chemical Co.) as a mass standard. The major fragments of the normal and deuterio \( p \)-bromophenylhydrazone are shown in Figure 2.

![Mass spectra](image)

**Fig. 2.**—(a) Mass spectra of \( p \)-bromophenylhydrazone of pyruvic acid (mol wt 257) and (b) \( p \)-bromophenylhydrazone of pyruvic acid -3-d.
The two isotopes of bromine 79 and 81 are present in a ratio of 50.5 to 49.5 and mass spectra of compounds containing bromine show ion peaks of equal intensity but differing by two mass units. Such isotopic clusters are seen for ions M⁺, (M-46)⁺, (M-86)⁺, (M-101)⁺, and (M-113)⁺ in the mass spectra of p-bromophenylhydrazone of pyruvic acid and for ions (M+1)⁺, [(M+1)-46]⁺, [(M+1)-87]⁺, [(M+1)-102]⁺, and [(M+1)-114]⁺ in the mass spectra of the deuterated compound. The ions (M-46)⁺, (M-86)⁺ in the nondeuterated compound and [(M+1)-87]⁺ in the deuterated compound represent the loss of the fragments CH₂O₂, C₃H₄O₂N, and C₃H₃D₀₂N, respectively (Fig. 3). These studies support the conclusion that the deuterium is located in the methyl group. From the intensity of the molecular ions, peak 257 and 259, the deuterium content of the pyruvate was calculated to be 82% which agrees well with the direct determination of deuterium by combustion. The small yet significant peak at mass 260 could arise only by contributions from 2D or C¹³D. Using the appropriate corrections, it is possible to calculate from the relative intensity of the 260 peak of the deuterio compound that about 6% of the deuterio pyruvate contains a —CHD₂ species and this would suggest that the enzyme-substrate complex undergoes a limited exchange with water.

In summary, the results reported here demonstrate that in the formation of the product pyruvate, the third hydrogen of the methyl group arises from water and not by intramolecular shift of a hydrogen from the ring of chorismate.

*Supported by a grant from the National Institutes of Health (GM 10384).
†Editorial note: Professor Rittenberg's letter of communication was written and signed by him on January 22, 1970, after his review of the manuscript in draft. The paper was then typed in its final form in accordance with suggestions made by Professor Rittenberg; it was therefore not actually transmitted to the PROCEEDINGS Office until after his untimely death on January 24.
In this circumstance, and at the request of the authors, we are glad to publish the following acknowledgment:

The authors would like to dedicate this paper To the memory of Professor David Rittenberg, a great teacher and a good friend.

We are also grateful to Dr. Laura Ponticorvo for her advice and help with the mass spectroscopy, and we thank Mrs. Irene Dym for her devoted assistance.

2 Srinivasan, P. R., Biochemistry, 4, 2860 (1965).