O^{18} STUDIES ON ANTHRANILATE HYDROXYLASE[1/]

— A NOVEL MECHANISM OF DOUBLE HYDROXYLATION —

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Anthranilate hydroxylase has been partially purified from cells of a pseudomonad and was shown to catalyze the formation of catechol from anthranilic acid (Kuno and Akaishi, 1961; Taniuchi et al., 1964). The reaction involves the consumption of one mole each of oxygen and of NADH[3/], with concomitant evolution of one mole each of CO_2 and ammonia per mole of the substrate utilized (Hosokawa et al., 1960; Taniuchi et al., 1964), (Eq. 1).

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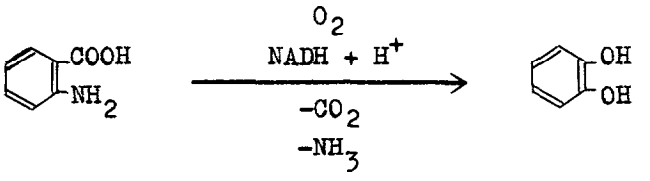
3/ Abbreviations: NADH, reduced nicotinamide adenine dinucleotide. CCMA, cis,cis-muconic acid.



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Many attempts to elucidate the mechanism of this complicated reaction have been made without success, presumably due to the instability and complex nature of this enzyme. In this communication, we wish to present experimental evidence indicating that both atoms of oxygen which were incorporated into catechol were derived from molecular oxygen. The results are in sharp contrast with the previously proposed mechanisms (Ichihara et al., 1962; Taniuchi et al., 1964) and indicate a possibility of a novel mechanism for the enzymic double hydroxylation.

Cell-free extracts were prepared from cells of Pseudomonas fluorescens No. 23 (ATCC 11250) as described previously (Taniuchi et al., 1964), and were then fractionated with ammonium sulfate. The fraction between 50 and 65 % saturation was used as a partially purified anthranilate hydroxylase in the following experiments. This enzyme preparation was practically free from the CCMA lactonizing enzyme (Sistrom and Stanier, 1954), but contains a sufficient amount of pyrocatechase, which converts catechol further to CCMA (Hayaishi et al., 1957). Hence, when this enzyme fraction was incubated in a standard reaction mixture[4/],

4/ Standard reaction mixture contains: anthranilic acid, 0.3 µmole; Tris acetate buffer, pH 7.3, 450 µmoles; ferrous sulfate, 0.3 µmole; NADH, 0.45 µmole; glucose, 200 µmoles; glucose dehydrogenase, 200 units; and enzyme, an amount which in a total volume of 3 ml causes a change in optical density of 0.01 to 0.1 per minute at 310 mµ.

catechol did not accumulate in an appreciable amount and instead, a stoichiometric amount of CCMA was formed judging from the increased ultraviolet absorbancy at 260 mμ.

When the reaction was carried out in an atmosphere of O_2^{18}, four atoms of oxygen in the isolated CCMA were found to be derived almost exclusively from molecular oxygen (Table I). On the other hand, when the reaction was carried out in the medium of H_2O^{18} and in an atmosphere of O_2^{16}, negligible amounts of O^{18} were found in the product. As shown in the control experiment when catechol was used as substrate approximately two atoms of atmospheric oxygen per mole of substrate were found to be incorporated into CCMA, in a good agreement with the earlier observation (Hayaishi *et al.*, 1957). Thus, it seems quite reasonable to conclude that the two oxygen atoms in catechol were derived entirely from atmospheric oxygen and not from the water molecule during the conversion of anthranilic acid to catechol.

Enzymic hydroxylation of the aromatic ring at two adjacent carbons was recently studied with kynurenic acid 7,8-hydroxylase (Taniuchi and Hayaishi, 1963). The reaction catalyzed by this enzyme proceeds from kynurenic acid *via* kynurenic acid 7,8-epoxide, a hypothetical intermediate, which is subsequently hydrated to form 7,8-dihydrokynurenic acid-7,8-diol. The latter compound is further converted to 7,8-dihydroxykynurenic acid by 7,8-dihydrokynurenic acid-7,8-diol dehydrogenase. By analogy, Ichihara *et al.*, (1962) and Taniuchi *et al.*, (1964) postulated the hypothetical mechanisms for the enzymic formation of catechol from anthranilic acid, according to which an epoxide is formed as a primary intermediate and subsequent hydration is involved.

According to these hypotheses one of the two oxygen atoms in catechol should originate from atmospheric oxygen and another

Table I.

Results of O^{18} experiment

Experiment I. Anthranilic acid (1.6 mmole) was incubated with 30 μmoles of NADH, 30 μmoles of $FeSO_4$, 83 mmoles of glucose, 1 mmole of glutathione, 43,200 units of glucose dehydrogenase[5], 75 units of pyrocatechase[6], 30 mmoles of Tris acetate buffer, pH 7.3, and 80 mg of anthranilate hydroxylase in a total volume of 200 ml in the atmosphere containing O^{18} with gentle mechanical shaking. After 150 minutes at 25°C, the reaction was almost complete as judged by the increase in absorbancy at 260 mμ. Then 30 ml of 2 N HCl were added to the chilled reaction mixture and CCMA was quickly extracted with 200 ml of ether twice. The combined ether solution was washed once with 200 ml of 10^{-2} M HCl and the ether was removed by evaporation. CCMA was recrystallized from absolute ethanol and its purity was established by melting point, ultraviolet absorption spectrum and elementary analyses. The recrystallized material was pyrolized by the method of Rittenberg and Ponticorvo (1956) and the O^{18} content was determined with an Atlas mass-spectrometer and a Hitachi mass-spectrometer. Experiment II. The experimental conditions were identical with those of experiment I except that the reaction was carried out for 120 minutes with 120 mg of anthranilate hydroxylase and without glutathione. Experiment III. The experimental conditions were identical with those of experiment I, except that catechol was used as substrate instead of anthranilic acid. Experiment IV and Experiment V. The experimental conditions were identical with those of experiment I and experiment II respectively, except that H_2O^{18} was used as a solvent and ordinary air was used as an atmosphere instead of O^{18} containing gas mixture.

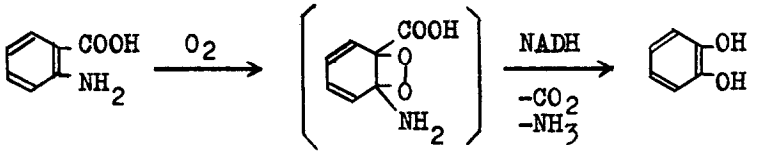
Exp. No.	Medium	Atom % excess of O^{18} in medium	Substrate	Atom % excess in CCMA	Atoms of O^{18} incorporated per mole	
					Observed	Theoretical+
I	O_2^{18}	1.34	Anth. A.	1.18	3.52	4.00
II	O_2^{18}	7.05	Anth. A.	5.94	3.37	4.00
III	O_2^{18}	1.27	Catechol	0.58	1.83	2.00
IV	H_2O^{18}	0.53	Anth. A.	0.02	0.15	0.00
V	H_2O^{18}	1.51	Anth. A.	0.05	0.13	0.00

+ Theoretical number of atoms incorporated per mole when four oxygen atoms were incorporated into CCMA from atmospheric oxygen during the conversion of anthranilate to the product, and two atoms were incorporated during the conversion of catechol to the product.

5/ Glucose dehydrogenase was purified by the method of Strecker (1955).

6/ Pyrocatechase was kindly donated by Dr. Y. Kojima of this laboratory.

from the water molecule. Contrary to these postulations, our results of O^{18} experiments demonstrate that both atoms of oxygen in catechol are exclusively derived from atmospheric oxygen. Since one mole each of oxygen and NADH is utilized for the formation of one mole of catechol, it is inferred that the reaction involves the direct incorporation of molecular oxygen into the substrate rather than two successive single hydroxylation reactions. Available evidence therefore indicates that two oxygen atoms, presumably in the same molecule, add to the double bond across the carbons 1 and 2, and the cyclic peroxide intermediate is reductively cleaved with concomitant release of ammonia and CO_2 (Eq. 2).



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Such a cyclic peroxide intermediate has been postulated in certain chemical reactions (Lunsford et al., 1955) and also in the pyrocatechase reaction (Hayaishi et al., 1957). The interpretation mentioned above is also consistent with the observation that anthranilate hydroxylase, unlike most other hydroxylases, requires ferrous ion for maximum activity. Further studies including the purification of the enzyme are now in progress to elucidate the intimate mechanism of this unique reaction.

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