# THE SOURCE OF OXYGEN IN THE PHENYLALANINE HYDROXYLASE AND THE DOPAMINE-β-HYDROXYLASE CATALYZED REACTIONS

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In recent years, several enzymatic hydroxylation reactions have been shown to utilize molecular oxygen as the source of the hydroxyl group (Mason, 1957). In a few cases balance studies have demonstrated that the hydroxylation reaction proceeds according to the general equation:

$$RH + O_2 + XH_2 \rightarrow ROH + H_2O + X \qquad (1)$$

where RH is the substrate which is hydroxylated and $XH_2$ is an electron donor. The hydroxylation reactions which fall into this class include the conversion of phenylalanine to tyrosine where the electron donor is a tetrahydropteridine (Kaufman, 1958), the conversion of Dopamine to norepinephrine where the electron donor is ascorbate (Levin, Levenberg and Kaufman, 1960), and the hydroxylation of kynurenine to 3-hydroxy kynurenine where TPNH appears to be the electron donor (Saito, Hayaishi, and Rothberg, 1957; de Castro, Price and Brown, 1956). Studies of the stoichiometry of other aerobic hydroxylation reactions will undoubtedly show that many of them follow this pattern.

It seems reasonable to conclude that in any hydroxylation reaction which follows the stoichiometry of equation 1, molecular oxygen is the source of the hydroxyl group (Kaufman, 1962a). At the present time, this conclusion, while reasonable, is largely unsupported by experimental data and it was thought desirable

to further test its validity by determining the source of the hydroxyl-oxygen in 2 hydroxylation reactions which proceed according to equation 1.

The present report describes the results of experiments which prove that molecular oxygen is utilized in the phenylalanine hydroxylation system as well as in the ß-Dopamine hydroxylation system, thus adding 2 more examples in support of the above generalization.

The $O^{18}$ content of various samples was determined using the Consolidated Engineering Corporation mass spectrometer (model 21-401). The $O^{18}$ content of the gas phase from the incubation tubes was determined from the ratio of mass 36 to masses 34 and 32 by a published method (Charalampous, 1960). Organic compounds were subjected to pyrolysis for 1 hour at 530° with dried $HgCl_2$ as catalyst according to the method of Rittenberg and Ponticorvo (1956). The $O^{18}$ content of the $CO_2$ formed was analyzed in the mass spectrometer and the enrichment in $O^{18}$ was obtained from the masses 44,45 and 46. The $O^{18}$ content of water of the incubation mixture was determined after distillation of an aliquot of the incubation mixture from the frozen state under reduced pressure (Eisenberg, unpublished). $H_2O^{18}$ containing 90.57 atom per cent excess $O^{18}$, and $O_2{}^{18}$ containing 98.0% atom per cent excess $O^{18}$ were purchased from the Weizmann Institute, Rehovoth, Israel.

The various components used in the phenylalanine hydroxylating system were prepared as already described (Kaufman, 1962b). The Dopamine ß-hydroxylase was purified by published methods (Levin, Levenberg and Kaufman, 1960).

For studying the source of the oxygen in the Dopamine ß-hydroxylase system, ß-phenylethylamine-$\alpha$-$C^{14}$, a substrate containing no oxygen was used. It has been shown that this

compound is hydroxylated to ß-phenylethanolamine by the purified enzyme which converts Dopamine to norepinephrine (Levin, and Kaufman, 1961). The radioactive ß-phenylethanolamine was isolated from the reaction mixture by ascending paper chromatography on Whatman 3 mm paper in n-butanol:ethanol:water (4:1:1). ß-phenylethanolamine was eluted from the paper with methanol and carrier ß-phenylethanolamine hydrochloride was added. The compound was crystallized from the methanol by the addition of ether to constant specific radioactivity.

The results of the $O^{18}$ analysis are shown in Table I. It can be seen that when the source of the $O^{18}$ was the atmosphere (experiments 1 and 2), the isolated product contained $O^{18}$; when the reaction was carried out in a medium of $H_2O^{18}$ with air as the gas phase, (experiment 3) no $O^{18}$ was found in the ß-phenylethanolamine.

Table I

$O^{18}$ Enrichment of ß-Phenylethanolamine

| Experiment | Medium | Atom per cent excess of $O^{18}$ in medium | Atom per cent excess of $O^{18}$ in product | | Per cent enrichment |
|---|---|---|---|---|---|
| | | | Found | Expected | |
| 1 | $O_2^{18}$ | 98.2 | 0.192 | 0.331 | 58.0 |
| 2 | $O_2^{18}$ | 92.5 | 0.059 | 0.097 | 60.8 |
| 3 | $H_2O^{18}$ | 31.6 | 0 | 0.149 | 0 |

The reaction mixtures contained potassium phosphate buffer, pH 6.5, 100 µmoles, fumarate, 50 µmoles, Worthington crystalline beef liver catalase, 450 units, ascorbate, 6 µmoles, ß-phenylethylamine-$\alpha$-$C^{14}$-HCl, 3.3 µmoles, purified hydroxylating enzyme, 0.23-0.37 mg protein, and either $H_2O^{16}$ or $H_2O^{18}$ to a final volume of 1.00 ml. The reaction vessels containing $H_2O^{16}$ were incubated at 35° for 1 hour under an atmosphere of 95.6 atoms per cent excess $O_2^{18}$, and those containing $H_2O^{18}$ were incubated under 100% $O_2^{16}$.

Results of studies on the phenylalanine hydroxylating system are shown in Table II. In most of the experiments, L-phenylalanine-U-$C^{14}$ was used as the substrate. The reaction was stopped by the addition of TCA, the protein removed by centrifugation, and the TCA was removed by successive extractions with ether. Carrier L-tyrosine was added to the reaction mixture, followed by sufficient water to dissolve all of the tyrosine at 100°C. The tyrosine which crystallized on cooling was recrystallized from water to constant specific activity.

Table II

$O^{18}$ Enrichment of Tyrosine

| Experiment (cofactor used) | Medium | Atom per cent excess of $O^{18}$ in medium | Atom per cent excess of $O^{18}$ in product | | Per cent enrichment |
|---|---|---|---|---|---|
| | | | Found | Expected* | |
| 1. $MMPH_4$ | $O_2{}^{18}$ | 80.4 | 0.69 | 1.17 | 59.0 |
| 2. $MMPH_4$ | $H_2O^{18}$ | 10.048 | 0.0024 | 0.103 | 2.3 |
| 3. Rat liver cofactor | $O_2{}^{18}$ | 79.9 | 0.1099 | 0.306 | 35.9 |
| 4. Rat liver cofactor | $H_2O^{18}$ | 10.575 | 0.0008 | 0.0474 | 1.7 |

The reaction mixtures contained the following components (in micromoles): potassium phosphate buffer pH 6.8, 100; TPNH, 0.5; L-phenylalanine, 6.0; glucose, 125; 2-amino-4-hydroxy-6-methyl-5,6,7,8-tetrahydropteridine ($MMPH_4$), 0.09; purified rat liver enzyme, 0.5 mg protein, purified sheep liver enzyme, 0.16 mg protein, glucose dehydrogenase 105 units and water up to 1.0 ml. In experiments 3 and 4, the purified rat liver cofactor after silica gel chromatography was used in place of the synthetic tetrahydropteridine. Incubations were carried out at 25° for 80-90 minutes.

* Assuming that only the phenolic oxygen is derived from the medium.

In one experiment, (Table II,3), the enzymatically synthesized tyrosine was separated from the other components in the reaction mixture by paper chromatography on Whatman 3 mm paper using 3°-butanol:methanol:water (4:5:1) as the developing solvent (Askonas, 1950). The tyrosine was eluted from the paper with water, mixed with carrier L-tyrosine, and then isolated as just outlined.

When the reaction was carried out in a medium of $H_2O^{18}$ with air as the gas phase, a negligible amount of $O^{18}$ was found in the tyrosine (Table II, experiment 2). On the other hand, if the reaction was carried out in normal water with $O_2^{18}$ in the atmosphere, (experiment 1) approximately 59% of the theoretical amount of $O^{18}$ was found in the product. When the purified rat liver hydroxylation cofactor was used in place of the synthetic tetrahydropteridine, the results were similar, although for reasons which are not known, the extent of incorporation was lower.

In view of the low values, the location of the $O^{18}$ in the tyrosine molecule was determined. An aliquot of the tyrosine (from experiment 1, Table II) was decarboxylated to tyramine and $CO_2$ by the procedure of Johnson and Daschavsky (1925) and the $CO_2$ was analyzed for $O^{18}$ content. None could be detected, proving that all of the $O^{18}$ in tyrosine was in the phenolic group.

The results of these experiments unequivocally establish $O_2^{18}$ as the source of the hydroxyl group in the products in both the side chain hydorxylating system (ß-Dopamine oxidase) and the aromatic ring hydroxylating system (phenylalanine hydroxylase). The reasons and possible mechanistic significance for the lower than theoretical enrichment in both systems remain obscure.

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