Aromatic Hydroxylation Mediated by Flavin Autoxidation: Lack of the NIH Shift

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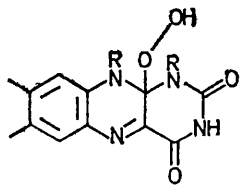
Summary Aromatic hydroxylations by two flavin monooxygenase model systems show low values of the NIH shift suggesting that these oxidations are free-radical processes.

TABLE. The NIH shift in aromatic hydroxylation with flavin and flavin model hydroxylases

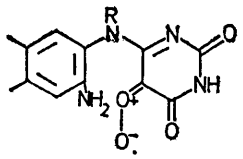
Substrate	Hydroxylating system	^3H or ^2H retention (%)
L-[4-^{3}H]Phenylalanine	Purified rat liver phenylalanine hydroxylase with tetrahydrobiopterin in phosphate buffer pH 6·9	87[a]
L-[4-^{3}H]Phenylalanine	1,3,10-trimethyl-5,10-dihydroalloxazine in 0·1 M H_2SO_4	8[b]
[2,6-$^2H_2$]Anisole	1,3,10-trimethyl-5,10-dihydroalloxazine in $MeCO_2H$	<1[c]
[2,6-$^2H_2$]Anisole	1,3,10-trimethyl-5,10-dihydroalloxazine in $MeCO_2H$–0·1 M H_2SO_4 (1 : 1, v/v)	<4[c]
[4-^{3}H]Anisole	Reduced flavine mononucleotide in phosphate buffer pH 7	3·5[d]
[4-^{3}H]Chlorobenzene	,,	1[e]

[a] 4-Hydroxyphenylalanine. This result is included for comparison with previous studies which used crude enzyme and 2-amino-4-hydroxy-6,7-dimethyltetrahydropterin. [b] 4-Hydroxyphenylalanine. Retention in 3-hydroxyphenylalanine 92·5%. [c] 2-Methoxyphenol. [d] 4-Methoxyphenol. Phenolic isomer distribution 2-, 59 : 3-, 12 : 4-, 29%. [e] Measured as 4-methoxychlorobenzene.

DESPITE extensive studies the mechanism of aromatic hydroxylation by flavin- and pterin-dependent monooxygenases remains a matter of controversy and speculation.[1-3] Although the site of oxygen activation is generally agreed to be the reduced flavin or pterin co-factor, the nature of the active oxidant is unclear. Mager and Berends,[4] from studies of the autoxidation of 1,3,10-trimethyl-5,10-dihydroalloxazine in the presence of aromatic substrates, have suggested a flavin peroxide (I),§ whilst Hamilton[1] in detailed discussions based on the oxenoid concept has proposed the carbonyl oxide intermediate (II). More recently even a flavin oxaziridine has been proposed as the active oxidant.[6]



(I)



(III)

We report the absence of a substantial NIH shift of tritium or deuterium during the hydroxylation by two model mono-oxygenase reactions (Table). In the Table are also presented for comparison the results of a typical NIH shift experiment with purified rat liver phenylalanine hydroxylase and the natural co-factor, tetrahydrobiopterin. Similar retention values have been obtained previously with the synthetic co-factor, 2-amino-4-hydroxy-6,7-dimethyltetrahydropterin.[7]

In a typical double labelling experiment with the reduced alloxazine model of Mager and Berends,[4] L-phenylalanine {0·05 μmol, 5·4 μCi of L-4-[^{3}H]phenylalanine with 1 μCi of L-[*U*-^{14}C]phenylalanine} was incubated with 1,3,10-trimethyl-5,10-dihydroalloxazine (20 μmol) in 0·1 M H_2SO_4 (0·5 cm^3) in air at 25 °C for 12 h. The mixture was separated by ion-exchange chromatography (A 120 B amino-acid column AA-15, 55 °C, 0·2M sodium citrate, pH 4·25, flow rate 60 cm^3 h^{-1} separated 2-, 3-, and 4-hydroxyphenylalanines).

Hydroxylation by the reduced flavin mononucleotide (H_2 with 10% Pd on charcoal) system[8] was brought about by stirring a suspension of substrate (0·1 cm^3) in a solution of reduced flavin (0·3 g) in phosphate buffer (300 cm^3, pH 7·0) at 20 °C in air. After 2 h the phenols were isolated by differential extraction and analysed by g.l.c.–m.s. under conditions described elsewhere.[9]

Clearly enzymes, such as phenylalanine hydroxylase, do not merely catalyse the spontaneous autoxidation of the reduced flavin or pterin co-factor since the latter occur with low values for the NIH shift. Further, if carbonyl oxide intermediates such as (II) are involved as the terminal oxidants in the model systems they must effect substrate oxidation mainly by non-oxenoid pathways which do not produce arene oxides. Presumably these oxidations are free-radical in nature. The hydroxyl radical as proposed by Mager and Berends[4] is one possibility but equally attractive oxidants would be flavin peroxy radicals or a radical form of (II). Blair and Pearson reached similar conclusions with a Udenfriend type model system, (Fe^{II} oxygen, and chemical reductant) employing tetrahydrobiopterin.[10]

(*Received*, 13*th August* 1975; *Com.* 938.)

§ The isomeric 4a-peroxide has also been suggested (refs. 1 and 5).

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