Hydroxylation of Phenylalanine by *Pseudomonas* sp.: Measurement of an Isotope Effect following the NIH Shift

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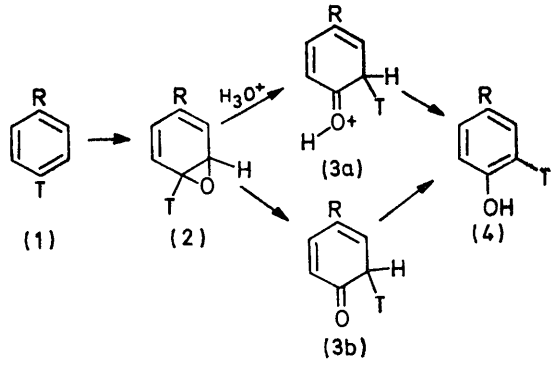
The conversion of [4-^{3}H]- and [4-^{3}H;3,5-$^2H_2$]phenylalanine into tyrosine by cultures of *Pseudomonas* sp. (ATCC 11299a) has been studied. Comparison of the tritium retentions observed during hydroxylation of the two precursors showed an isotope effect, $k_D/k_T = 2{\cdot}8 \pm 0{\cdot}1$ ($k_H/k_D = 10 \pm 1$), for the final aromatisation reaction following the NIH shift. This value is compared with those of likely chemical models for the biochemical process.

EXTENSIVE investigations,[1] mainly in the National Institutes of Health (Maryland), have demonstrated that biological hydroxylation of aromatic substrates may involve migration of hydrogen (the NIH shift) from the site of attack to a neighbouring carbon atom. A favoured mechanism [2] involves conversion of the substrate, by a mono-oxygenase system, into an arene oxide (2) which isomerises *via* the keto-form of a phenol (3a or b) into the phenol itself (4). The NIH shift, here



exemplified with tritium, occurs as a consequence of epoxide ring opening and may be concerted or, as currently preferred for chemical systems,[3] stepwise. Often, with biological hydroxylations, high retention of tritium in the phenol (4) is observed. For example, Guroff and Daly showed [4] that the hydroxylase systems of *Pseudomonas* and rat livers convert [4-^{3}H]-phenylalanine (5; R = H) into [3-^{3}H]tyrosine (6; R = H) with >90% tritium retention. They demonstrated convincingly that high retention resulted from the operation of a kinetic isotope effect in the enolisation step, (3a or b) ⟶ (4), since tritium was largely retained during hydroxylation of both [4-^{3}H]- and [3-^{3}H]-phenylalanine. This result eliminates the alternative possibilities that (*a*) tritium migration (from C-4 to C-3) is concerted with hydrogen removal (from C-3) or (*b*) an enzyme removes, stereospecifically, hydrogen from the newly formed chiral centre (C-3) in the intermediate (3a or b). We now record experiments with *Pseudomonas* cultures designed to measure the kinetic isotope effect involved in this enolisation process.

If we assume complete migration of tritium during hydroxylation, then the observed retention of tritium in the phenolic product should provide directly the required value of k_H/k_T. However, the calculated value would be extremely sensitive to small experimental errors; for example, retentions of 95, 96, and 97% would correspond to values of 19, 24, and 32 respectively for k_H/k_T. Use of deuterium in place of tritium is helpful but the retention of deuterium would still be inconveniently high. Using the Swain [5] relationship, $k_H/k_T = (k_H/k_D)^{1{\cdot}442}$, if, for example, $k_H/k_T = 24$ (96% retention of tritium), then $k_H/k_D = 9$ (90% retention of deuterium). Moreover, measurement of the deuterium content of metabolites requires isolation without substantial dilution with unlabelled material. This is seldom easy with intact organisms, especially if the product must be carefully purified by methods which do not cause any loss of label by exchange (see later). Instead we elected [6] to measure the smaller quantity k_D/k_T since, for $k_H/k_T = 24$, $k_D/k_T = 2{\cdot}6$, and the expected tritium retention (72%) falls conveniently mid-way between the extreme values of 50 (no isotope effect) and 100% (infinite isotope effect).

Labelled specimens of phenylalanine were prepared as follows. 4-Iodotoluene was treated successively with lithium metal and [^{3}H]water to provide [4-^{3}H]toluene which was brominated photochemically to give [4-^{3}H]-benzyl bromide. This was used to prepare DL-[4-^{3}H]-phenylalanine (5; R = H) by standard methods.[7] 4-Aminotoluene hydrochloride was converted,[8] by repeated exchange in deuterium oxide, into 4-amino-[3,5-$^2H_2$]toluene. Diazotisation and treatment with potassium iodide gave 4-iodo[3,5-$^2H_2$]toluene which was converted, as before, *via* [4-^{3}H;3,5-$^2H_2$]toluene into DL-[4-^{3}H;3,5-$^2H_2$]phenylalanine (5; R = D). Each step in the syntheses was monitored (n.m.r. and mass spectra) in advance, using, when necessary, deuterium in place of tritium. The position of tritium in the phenylalanines was confirmed by degradation (Table 1). Each

[1] G. Guroff, J. W. Daly, D. M. Jerina, J. Renson, B. Witkop, and S. Udenfriend, *Science*, 1967, **157**, 1524.
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[3] G. J. Kasperek, T. C. Bruice, H. Yagi, and D. M. Jerina, *J.C.S. Chem. Comm.*, 1972, 784.
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[6] *Cf.* W. R. Bowman, I. T. Bruce, and G. W. Kirby, *Chem. Comm.*, 1969, 1075.
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[8] W. C. Ripka and D. E. Applequist, *J. Amer. Chem. Soc.*, 1967, **89**, 4035; A. P. Best and C. L. Wilson, *J. Chem. Soc.*, 1956, 239.

was treated with alkali and dimethyl sulphate to give cinnamic acid, which was then oxidised to benzoic acid. Treatment with hydrazoic acid gave aniline, which was acetylated and brominated to yield 4-bromoacetanilide. Finally the labelled benzoic acids were converted into their 3,5-dinitro-derivatives.

TABLE 1

Degradation of cinnamic acids (relative molar activity 1·00) derived from DL-[4-^{3}H]- and [4-^{3}H;3,5-$^2H_2$]-phenylalanine

Labelling pattern	Benzoic acid	3,5-Dinitrobenzoic acid	Acetanilide	4-Bromoacetanilide
[4-^{3}H]	1·02	1·00	1·00	<0·01
[4-^{3}H;3,5-$^2H_2$]	0·99	0·99	1·00	<0·01

The hydroxylation of phenylalanine was studied by use of a *Pseudomonas* strain (ATCC 11299a) previously adapted to the use of the amino-acid as sole carbon source.[9] The intact organism, rather than purified hydroxylase preparations,[4,10] was employed since the latter might conceivably [6] have lacked an enzyme controlling the enolisation step, (3a or b) ⟶ (4). The tritiated phenylalanines (see before) were each mixed with DL-[*carboxy*-^{14}C]phenylalanine to provide samples of known ^{3}H : ^{14}C ratio. The radioactive precursors were administered to *Pseudomonas* cultures growing on unlabelled phenylalanine and mineral salts. Radioactive tyrosine was detectable in the medium after *ca.* 0·5 h and had become the major radioactive constituent after *ca.* 6 h. When the conversion of phenylalanine into tyrosine was judged to be adequate (*ca.* 6 and *ca.* 17% conversion in two series of experiments) unlabelled L-tyrosine was added, in dilute hydrochloric acid, to the medium, and the cells were removed by centrifugation. The supernatant (readjusted to pH 6—7) was concentrated and set aside to allow crystallisation of tyrosine, which was then recrystallised to constant specific activity and ^{3}H : ^{14}C ratio. Repeated recrystallisation of purified material did not cause loss, by exchange, of tritium. The entire experiment was carried out near pH 6, conditions which were not expected [11] to cause removal of tritium from positions *ortho* to the phenolic hydroxy-group of tyrosine. This was borne out by the high retention of tritium observed in tyrosine derived from [4-^{3}H]phenylalanine. The labelled tyrosine specimens were converted, with alkali and dimethyl sulphate, into 4-methoxycinnamic acid without loss of ^{3}H or ^{14}C. Permanganate oxidation of the cinnamic acid gave 4-methoxybenzoic acid with complete (>98%) removal of ^{14}C and retention of ^{3}H. Treatment of the tyrosine with hot hydrochloric acid, under conditions known [11] to remove tritium only from the 3- and 5-positions, gave material having the same ^{14}C activity and containing negligible (<1%) amounts of tritium. Thus hydroxylation of phenylalanine had occurred without ' scrambling ' of the radiolabels and with the characteristic NIH shift of tritium. The results are tabulated (Table 2).

In accord with earlier work [4] using cell-free systems, hydroxylation of [4-^{3}H]phenylalanine (5; R = H) by the intact organism proceeded with high (*ca.* 95%) migration and retention of tritium. In contrast, with the deuteriated precursor (5; R = D) a much lower (*ca.* 74%) retention of tritium was observed. Similar results were obtained from two independent sets of experiments involving widely different % conversions of precursor into product. These findings show that migration of tritium is virtually complete (certainly >95%) and retention thereafter is dictated by a large kinetic isotope effect. Counting errors were <1% but systematic errors involved in the measurement of counting efficiencies may inflate this figure. The small differences observed between the two sets of experiments are not considered significant and retention values of 95 ± 1% for (5; R = H) and 74 ± 1% for (5; R = D) will be used for discussion. If we assume complete migration of tritium during hydroxylation of (5; R = D) then 74 ± 1% retention of tritium corresponds to k_D/k_T = 2·8 ± 0·1. If we accept [12,13] the Swain relationship and ignore possible small secondary isotope effects [13,14] the corresponding k_H/k_D ratio is 10 ± 1. Chemical analogies for the enolisation step (3a) ⟶ (4) are available from work on the acid-catalysed exchange of labelled, activated aromatic systems.[13-15] Values in the range 5—9 are typical. For example,[15] aromatisation of the *ortho*-protonated anisole (7), a reasonable model for (3a), proceeds with a rate ratio, k_H/k_D = 7·2. Our value, 10 ± 1, for the *Pseudomonas* hydroxylase system, is higher but the alternative process (3b) ⟶ (4) may be more relevant. Kasperek and Bruice have recently

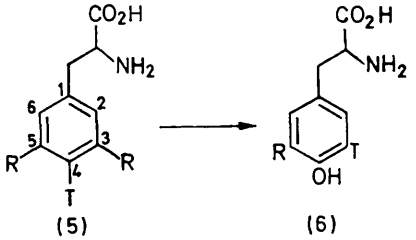
TABLE 2

Incorporation of DL-phenylalanine into L-tyrosine by Pseudomonas (ATCC 11299a)

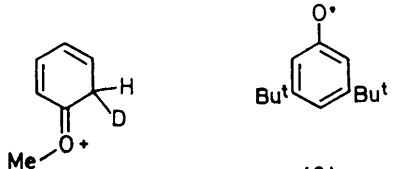
Labelling pattern	^{3}H : ^{14}C Ratios: Precursor	Tyrosine	4-Methoxycinnamic acid	Incorpn. (%)	^{3}H Retention (%)
[4-^{3}H;*carboxy*-^{14}C]	5·43	5·20	5·20	16·5	95·8
[4-^{3}H;*carboxy*-^{14}C]	4·23	3·99	3·96	6·2	94·0
[4-^{3}H;3,5-$^2H_2$;*carboxy*-^{14}C]	5·88	4·38	4·45	18·2	75·1
[4-^{3}H;3,5-$^2H_2$;*carboxy*-^{14}C]	5·01	3·70	3·67	6·0	73·6

[9] G. Guroff and T. Ito, *J. Biol. Chem.*, 1965, **240**, 1175.
[10] G. Guroff and C. A. Rhoads, *J. Biol. Chem.*, 1967, **242**, 3641.
[11] G. W. Kirby and L. Ogunkoya, *J. Chem. Soc.*, 1965, 6914.
[12] For recent discussion, see M. J. Stern and P. C. Vogel, *J. Amer. Chem. Soc.*, 1971, **93**, 4664.
[13] A. J. Kresge and Y. Chiang, *J. Amer. Chem. Soc.*, 1967, **89**, 4411.
[14] J. L. Longridge and F. A. Long, *J. Amer. Chem. Soc.*, 1967, **89**, 1292.
[15] A. J. Kresge, *Discuss. Faraday Soc.*, 1965, **39**, 48.

shown [16] that 1,2-epoxy-1,2-dihydrobenzene (2; R = H, H in place of T) undergoes acid-catalysed isomerisation, presumably *via* (3a; R = H, H in place of T), to give phenol. At low acidities (pH > 6), however, an alternative route, involving (3b; R = H, H in place of T), dominates the isomerisation. An estimate for the isotope effect associated with the process (3b) ⟶ (4) is



(5) (6)



(7) (8)

available [17] from kinetic studies on the oxidative coupling of phenols. Comparison of the rates of dimerisation of the radical (8) and its [2,4,6-$^2H_3$]-derivative in neutral benzene gave an apparent isotope effect of 10 ± 2. In the presence of toluene-4-sulphonic acid no significant rate difference was observed. The authors conclude that, in the absence of acid, the rate-determining step is the enolisation of a keto-form of the C–C coupled, phenolic product. Thus the hydroxylation of phenylalanine by *Pseudomonas* is at present best interpreted by the sequence (1) ⟶ (2) ⟶ (3b) ⟶ (4) and, even with intact organisms, there is no indication that the last two steps are enzymically, and thereby stereochemically, controlled.

EXPERIMENTAL

Counting Methods.—Activities (^3H and ^{14}C) were measured with a Beckman type CPM-100 liquid scintillation spectrometer calibrated with [2,3-$^3H_2$]- and [1-^{14}C]-hexadecane. Phenylalanine and tyrosine (*ca.* 0·5 mg) were dissolved, in counting vials, in ethanolic hydrogen chloride and the solvent was evaporated *in vacuo* (over KOH) at room temperature. The residues were dissolved in dimethylformamide (1 ml) and toluene-based scintillator solution (10 ml) for counting. A control experiment showed that this procedure did not remove tritium from [3-^3H]tyrosine.

DL-[4-^3H]*Phenylalanine.*—4-Iodotoluene (sublimed) (3·27 g) in dry ether (40 ml) was stirred with lithium (0·25 g) under nitrogen for 3 h. Tritiated water (0·5 ml; 3·6 mCi mmol^{-1}) was added and, after 20 min, the mixture was diluted with water. The aqueous layer was extracted with ether (3 × 10 ml) and the combined ethereal solutions were dried (Na_2SO_4) and concentrated by distillation (ambient pressure) through a short fractionating column. Carbon tetrachloride (20 ml) was added to the concentrate and the last traces of ether were distilled off. The concentration of [4-^3H]toluene (90% yield) in the remaining carbon tetrachloride was measured from the n.m.r. spectrum by comparison with a standard solution.

The foregoing solution was irradiated with tungsten lamps while being treated dropwise with bromine (0·8 ml) in carbon tetrachloride (20 ml) at such a rate that bromine did not persist in the mixture. The resulting solution was washed with water (3 × 10 ml), dried (Na_2SO_4), and evaporated to *ca.* 3 ml. Distillation at ambient pressure gave [4-^3H]benzyl bromide (1·09 g). This was converted by standard methods [7] *via* 5-[4-^3H]benzyl-3-phenylhydantoin into DL-[4-^3H]phenylalanine (1·47 mCi mmol^{-1}).

DL-[4-^3H;3,5-$^2H_2$]*Phenylalanine.*— 4-Aminotoluene hydrochloride (3·5 g) was exchanged [8] (48 h under reflux in nitrogen) with five successive batches of deuterium oxide (2·5 ml per batch). Diazotisation and treatment with potassium iodide gave 4-iodo[3,5-$^2H_2$]toluene, which was converted into DL-[4-^3H; 3,5-$^2H_2$]phenylalanine as before.

Feeding Experiments.—The *Pseudomonas* sp (ATCC 11299a, NCIB 9289) was grown on the recommended [10] phenylalanine–mineral salts medium (pH 6·5—7·0) without yeast extract but with iron(II) diammonium bis-sulphate (10^{-4}%) at 30 °C with shaking. Batches (100 ml) of the medium, containing non-radioactive L-phenylalanine (200 mg), were inoculated with suspensions (1 ml) of *Pseudomonas* previously grown on the same medium for 24 h. After 36 h the labelled phenylalanine (typically 50 μCi ^3H and 10 μCi ^{14}C) was added and the course of metabolism followed by paper chromatography and radioactive scanning. After *ca.* 6 h (prolonged incubation leads to the disappearance of tyrosine), non-radioactive L-tyrosine (70 mg) in 4N-hydrochloric acid (5 ml) was added and the cells were centrifuged off. The supernatant was adjusted to pH 5·5—6·0 and evaporated to *ca.* 5 ml. The crude tyrosine which separated was collected and dissolved in dilute hydrochloric acid. The solution was adjusted quickly to pH *ca.* 6 and the tyrosine allowed to crystallise slowly. Recrystallisation was repeated several times although, generally, little change in activity was observed after the first crystallisation.

Degradation of Phenylalanine and Tyrosine.—The amino-acids were treated with an excess of aqueous sodium hydroxide and dimethyl sulphate, first at 0 and then at 100 °C, to afford the corresponding cinnamic and 4-methoxycinnamic acids. These were oxidised with potassium permanganate in hot aqueous sodium carbonate to give, respectively, benzoic and 4-methoxybenzoic acid. Labelled tyrosine was heated in 4N-hydrochloric acid at 100 °C for 16 h to remove tritium from the 3- and 5-positions. Benzoic acid was converted [18] (NaN_3–conc. H_2SO_4) into aniline and thence acetanilide and 4-bromoacetanilide. Nitration (fuming HNO_3–conc. H_2SO_4) of the benzoic acid gave 3,5-dinitrobenzoic acid.

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