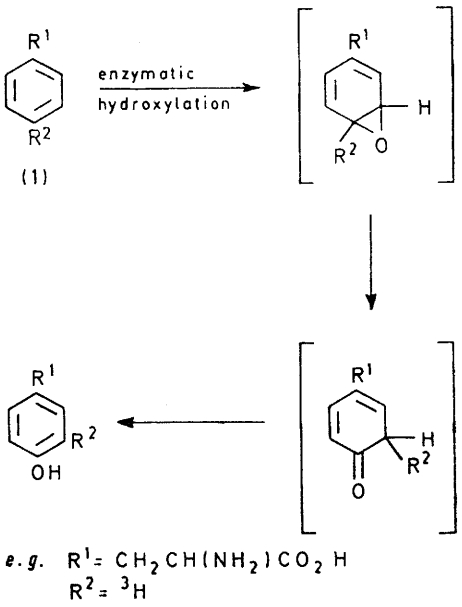
Non-enzymatic, Tetrahydrobiopterin-mediated Hydroxylation of Phenylalanine

By **John A. Blair** and **Anthony J. Pearson,*** Department of Chemistry, University of Aston in Birmingham, Gosta Green, Birmingham B4 7ET

Product yields are reported for the tetrahydrobiopterin-mediated, non-enzymatic hydroxylation of phenylalanine under a variety of conditions, and compared with reactions involving Fenton's reagent. Hydroxyl radicals are shown to be partially involved, whilst the hydroperoxyl radical appears to be unreactive towards hydroxylation. A low N.I.H. shift indicates the reactive entity to be free radical in nature, and the involvement of iron(II)–oxygen complexes is considered.

DURING attempts to elucidate the mechanism of activation of molecular oxygen in enzymatic hydroxylation of aromatic substrates, many model chemical systems have been studied. Fenton's reagent (hydrogen peroxide–ferrous ion) is known [1] to hydroxylate the benzene ring through generation of the hydroxyl radical. The Udenfriend system [2] (aqueous ascorbate–ferrous ion–oxygen, usually containing EDTA), which is essentially similar to the reaction reported here, was shown to lead to non-specific hydroxylation of the aromatic nucleus, and the exact nature of the hydroxylating entity is still unknown. Mason and Onoprienko [3] have shown, using ^{18}O-labelled gas, that the hydroxy-group introduced during this reaction is derived almost entirely from molecular oxygen, and Mason [4] has suggested that the mechanism is homolytic rather than heterolytic. Breslow and Lukens [5] proposed that hydrogen peroxide, generated during ascorbate autoxidation, was decomposed to hydroxyl radicals, these being the predominant hydroxylating species, but Grinstead [6] and Hamilton *et al.*[7] showed that inclusion of catalase in the system did not reduce the total hydroxylation yield. Grinstead did suggest at least partial participation of hydrogen peroxide, since a change in relative yields of different products occurred on addition of catalase. Norman and Radda [8] have also concluded that the hydroxyl radical is not the major reactive entity, and Norman and Lindsay Smith [9] have suggested the involvement of complexes of ascorbate, iron(II), and oxygen. The mechanism proposed by Hamilton,[10] in which such complexes decompose to yield the reactive oxene, :Ö:, is open to some doubt since, by analogy with carbenes, such an entity might be expected to react with carbon–carbon double bonds to produce an epoxide. It is known that formation of such intermediates during, for example, aromatic *para*-hydroxylation results in an N.I.H. shift, in which the original *para*-substituent (*e.g.* tritium) migrates to the adjacent position as outlined in Scheme 1, whilst the Udenfriend system does not give a significant N.I.H. shift (see review by Jerina and references therein [11]).

It has been shown [12] that ascorbate can be replaced by tetrahydropterin derivatives in the Udenfriend system,



SCHEME 1 N.I.H. shift during *para*-hydroxylation of *para*-disubstituted benzenes

and this will lead to hydroxylation of phenylalanine, though an extensive study of product yields has not appeared. Since tetrahydropterins are known [13] to be active cofactors in the phenylalanine hydroxylase system, it might be anticipated that the chemical reactions would furnish a useful mechanistic model. However, the lack

[1] (*a*) J. H. Baxendale, M. G. Evans, and G. S. Park, *Trans. Faraday Soc.*, 1946, **42**, 155; (*b*) J. H. Mertz and W. A. Waters, *J. Chem. Soc.*, 1949, S15; (*c*) W. T. Dixon and R. O. C. Norman, *Nature*, 1962, **196**, 891; (*d*) J. R. Lindsay Smith and R. O. C. Norman, *J. Chem. Soc.*, 1963, 3897.

[2] B. B. Brodie, J. Axelrod, P. A. Shore, and S. Udenfriend, *J. Biol. Chem.*, 1954, **208**, 741.

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[5] R. Breslow and L. N. Lukens, *J. Biol. Chem.*, 1960, **235**, 292.

[6] R. R. Grinstead, *J. Amer. Chem. Soc.*, 1960, **82**, 3472.

[7] G. A. Hamilton, R. J. Workman, and L. Woo, *J. Amer. Chem. Soc.*, 1964, **86**, 3390.

[8] R. O. C. Norman and G. K. Radda, *Proc. Chem. Soc.*, 1962, 138.

[9] R. O. C. Norman and J. R. Lindsay Smith in ' Oxidases and Related Redox Systems,' eds. T. E. King, H. S. Mason, and M. Morrison, Wiley, New York, 1964, vol. 1, p. 131.

[10] G. A. Hamilton, *J. Amer. Chem. Soc.*, 1964, **86**, 3391.

[11] D. M. Jerina, *Chem. Tech.*, 1973, 120.

[12] A. Bobst and M. Viscontini, *Helv. Chim. Acta*, 1966, **49**, 884; W. F. Coulson, M. J. Powers, and J. B. Jepson, *Biochim. Biophys. Acta*, 1970, **222**, 606.

[13] S. Kaufman, *J. Biol. Chem.*, 1959, **234**, 2683.

of N.I.H. shift given by all these model systems compared to the enzyme reaction indicates that this is not so, and in fact the only chemical reagents giving comparable migrations are those which implicate the cation OH^+ (*e.g.* peroxytrifluoroacetic acid).[14]

With regard to phenylalanine hydroxylations, Nofre *et al.*[15] have reported that Fenton reagent oxidation gives *o*-, *m*-, and *p*-tyrosines, 2,3-, 2,5-, and 3,4-dihydroxyphenylalanines, aspartic acid, alanine, glycine, and three unknown compounds; monohydroxylation products were predominant. Dalgliesh [16] has observed *o*- and *p*-tyrosines, 2,3-, 2,5-, and 3,4-dihydroxyphenylalanines, hydroquinone, and possibly catechol during the Udenfriend (ascorbate) reaction, and concluded that dihydroxylated compounds arise by further reaction of the tyrosines.

This paper reports the results of some more detailed studies of product analysis, under a variety of conditions, for hydroxylation of phenylalanine by the tetrahydrobiopterin–ferrous ion–oxygen system and Fenton's reagent, and also the N.I.H. shifts given by these reactions.

RESULTS AND DISCUSSION

Products and Yields.—Table 1 shows typical R_F values of authentic materials corresponding to anticipated products, in two solvent systems which give reasonably good separation. System (1) was chosen for yield determinations because (a) it separated products into types, *e.g.* monohydroxylated, dihydroxylated, and fragmentation, and (b) although in certain cases it appears to give poorer separation than (2), the spots in the latter were actually more diffuse and sometimes very distorted, particularly at high R_F values.

TABLE 1

Typical R_F values for possible products of phenylalanine hydroxylation

Compound (authentic)	R_F Solvent (1)	R_F Solvent (2)
Phenylalanine	0·66	0·74
o-Tyrosine	0·57	0·73
m-Tyrosine	0·51	0·64
p-Tyrosine	0·47	0·62
2,3-Dihydroxyphenylalanine	0·43	0·47
2,5-Dihydroxyphenylalanine	0·38	0·38
3,4-Dihydroxyphenylalanine	0·36	0·30
Aspartic acid	0·27	0·15
Alanine	0·27	0·57
Glycine	0·26	0·36

Solvent (1) n-butanol–acetic acid–water (4 : 1 : 5 v/v, organic phase after 24 h equilibration). Solvent (2) phenol–water (9 : 1 w/v).

The products retaining the carboxy-group observed in the ^{14}C experiments (see Experimental section) were *o*- and *p*-tyrosines, 3,4-dihydroxyphenylalanine, and aspartic acid. *m*-Tyrosine could not be detected by autoradiography even after prolonged exposure so any *minor* yield was included in that of *o*-tyrosine (though it separates quite well from *o*- and *p*-tyrosines). 2,3-Dihydroxyphenylalanine was not found, and 2,5-dihydroxyphenylalanine chromatographed too closely to the 3,4-isomer to allow separate determinations, hence all three were measured as a combined yield (Dopas). The presence of 2,5-Dopa was inferred from the relatively intense Dopa spot on autoradiograms from hydroxylation of [4-^{3}H]phenylalanine. An unidentified compound (Unknown 1) eluted between aspartic acid and Dopa was observed and determined, and radioactivity was also found at R_F values below that of aspartic acid. Some of the better autoradiograms indicated possibly up to three unidentified products in this region, and these were determined as a combined yield (Unknown 2).

The yields of products, including unchanged phenylalanine, are presented in Table 2 for the Fenton reaction and tetrahydrobiopterin system under a variety of conditions. It can be seen that the relative yields are susceptible to slight changes in reaction conditions. Reaction I showed aspartic acid as the major product and its identity was confirmed by chromatography in solvent (2) which separates it from alanine and glycine [these three are eluted very closely in solvent (1)]. This variation in relative yields makes it impossible to identify the reactive entity in the tetrahydrobiopterin system by direct comparison with Fenton's reagent ($OH^\bullet$ radicals) or any other well characterised reaction. However, the addition of catalase gives rise to some interesting effects. Hydroxylation by Fenton's reagent is completely eliminated by the enzyme (reaction G), any minor yields probably being due to radiochemical impurities. On the other hand, there is a slight increase in total hydroxylation by the tetrahydrobiopterin system, together with a significant change in product ratios, in the presence of EDTA (compare reactions A and B) whilst in the absence of the ligand (C and D) a reduction in total yield occurs without significant change in relative yields (for convenience, Table 3 shows yields relative to *p*-tyrosine). These effects are not yet fully understood, and the role of EDTA requires more rigorous investigation. Although the solutions are unbuffered (pH *ca.* 2·5), addition of the small amount of catalase used results in a negligible pH shift (<0·1 pH units), so that the effects observed are not due to changes in acidity. It is clear that in the absence of EDTA the hydrogen peroxide generated by tetrahydrobiopterin autoxidation [17] is partially responsible for hydroxylation *via* a Fenton-type reaction. Comparison of total yields from C and D indicate that this accounts for *ca.* 30% of the total hydroxylation. It is possible that hydroxyl radicals may be generated in an alternative way

[14] D. M. Jerina, J. W. Daly, W. Landis, B. Witkop, and S. Udenfriend, *J. Amer. Chem. Soc.*, 1967, **89**, 3347.

[15] C. Nofre, A. Cier, C. Michon-Saucet, and J. Parnet, *Compt. rend.*, 1960, **58**, 214; C. Nofre, J. P. Charrier, and A. Cier, *Bull. Soc. chim. Biol.*, 1963, **45**, 913.

[16] C. E. Dalgliesh, *Arch. Biochem. Biophys.*, 1955, **58**, 214.

[17] J. A. Blair and A. J. Pearson, *J.C.S. Perkin II*, 1974, 80; A. J. Pearson, *Chem. and Ind.*, 1974, 233.

and, even though catalase is known [18] to scavenge this species, its involvement cannot be discounted on the basis of incomplete suppression of hydroxylation by the enzyme. The change in relative yields in the presence of being listed in Table 4. Under no conditions was any inhibition observed, but rather a slight enhancement which is possibly due to slowing down of the tetrahydrobiopterin autoxidation reaction, thereby leading to the

TABLE 2

Analysis of reaction mixtures from phenylalanine hydroxylation with tetrahydrobiopterin system and Fenton's reagent

(a) Tetrahydrobiopterin system

	Reaction				
Product	A *	B *	C †	D ‡	E *
Unchanged phenylalanine	59·4 ± 2·5	53·4 ± 2·0	57·3 ± 0·6	69·3 ± 0·6	85·5 ± 1·5
o-Tyrosine	8·0 ± 1·5	5·2 ± 1·0	5·7 ± 0·5	3·7 ± 0·3	3·3 ± 1·0
p-Tyrosine	5·7 ± 0·4	9·2 ± 0·5	7·9 ± 0·2	5·5 ± 0·1	3·4 ± 0·3
Total Dopas	3·9 ± 0·3	13·8 ± 0·6	10·4 ± 0·4	7·9 ± 0·4	2·6 ± 0·2
Unknown 1	1·9 ± 0·4	4·8 ± 1·0	3·6 ± 0·2	2·6 ± 0·1	1·6 ± 0·4
Aspartic acid	6·2 ± 0·6	4·0 ± 0·5	4·2 ± 0·2	2·8 ± 0·3	1·5 ± 0·3
Unknowns 2	14·8 ± 0·6	9·1 ± 0·5	11·0 ± 0·1	8·2 ± 0·3	2·0 ± 0·3

(b) Fenton reactions

	F *	G §	H *	I *
Unchanged phenylalanine	83·5 ± 2·7	98·2	37·3 ± 0·5	3·0 ± 0·2
o-Tyrosine	5·5 ± 1·5	0·9	18·1 ± 0·8	12·1 ± 1·0
p-Tyrosine	4·2 ± 0·7	0·3	13·4 ± 0·5	3·5 ± 0·3
Total Dopas	2·8 ± 0·4	0·4	9·0 ± 0·4	6·4 ± 0·4
Unknown 1	0·8 ± 0·1	0·1	2·2 ± 0·2	2·6 ± 0·3
Aspartic acid	2·0 ± 0·4	0·1	10·3 ± 0·4	52·1 ± 0·5
Unknowns 2	1·6 ± 0·1	0·1	9·3 ± 0·4	20·4 ± 0·5

* Mean of two determinations ± range. † Mean of four determinations ± S.E.M. (standard error of mean). ‡ Mean of three determinations ± S.E.M. § One determination only.

Reaction conditions: A, 1·56mM-phenylalanine, 0·6mM-Fe^{2+}, 5mM-EDTA, 12·8mM-tetrahydrobiopterin; B, as A + catalase (0·2 mg cm^{-3}); C, as A without EDTA; D, as C + catalase (0·2 mg cm^{-3}); E, as C without added Fe^{2+}; F, 1·56mM-phenylalanine, 0·6mM-Fe^{2+}, 5mM-EDTA, 6·75mM-H_2O_2; G, As F + catalase (0·2 mg cm^{-3}); H, 0·69mM-phenylalanine, 0·67mM-Fe^{2+}, 5mM-EDTA, 13·0mM-H_2O_2; I, as F but using 0·5M-H_2O_2.

TABLE 3

Effect of catalase on product distribution from tetrahydrobiopterin-mediated phenylalanine hydroxylation

	Yields relative to *p*-tyrosine			
Reaction / Product	A *	B *	C †	D ‡
p-Tyrosine	1·00	1·00	1·00	1·00
o-Tyrosine	1·40 ± 0·31	0·57 ± 0·10	0·72 ± 0·07	0·67 ± 0·06
Total Dopas	0·68 ± 0·07	1·50 ± 0·08	1·32 ± 0·06	1·44 ± 0·08
Unknown 1	0·33 ± 0·07	0·52 ± 0·11	0·46 ± 0·03	0·47 ± 0·02
Aspartic acid	1·09 ± 0·14	0·44 ± 0·06	0·53 ± 0·03	0·51 ± 0·06
Unknowns 2	2·60 ± 0·23	0·99 ± 0·06	1·39 ± 0·04	1·49 ± 0·07

Reaction conditions as in Table 2.

* Mean of two determinations ± range. † Mean of four determinations ± S.E.M. ‡ Mean of three determinations ± S.E.M.

EDTA indicate that the major hydroxylating agent is not the hydroxyl radical.

We have published evidence [17] that the chain carrier during autoxidation of tetrahydrobiopterin is the hydroperoxyl radical $HO_2^{\bullet}$ (at low pH) or the superoxide anion radical, O_2^{-} (at neutral pH and higher). Although this species is reported [1d] to be relatively unreactive in aromatic hydroxylation, it may be implicated in other ways, *e.g.* as a complex with iron(II). In order to eliminate this possibility, the effect of superoxide dismutase on total hydroxylation yields was determined, the results

* The autoxidation of tetrahydrobiopterin in 0·1M-sodium phosphate buffer, pH 7, showed a 50% reduction in rate when superoxide dismutase was incorporated.

increased availability of tetrahydrobiopterin in the hydroxylation reaction. In particular, reactions (5) and (6) in Table 4 utilised conditions of pH and oxygen concentration under which the enzyme was observed to inhibit the autoxidation,* so that superoxide does not appear to be active in the hydroxylation reaction.

N.I.H. Shift as a Criterion for Free-radical Hydroxylation.—The tritium retentions, after correction for radiochemical impurity, which were observed for the Fenton and tetrahydrobiopterin systems are given in Table 5.

[18] A. von Henglein, W. Karmann, W. Roebke, and G. Beck, *Makromol. Chem.*, 1966, **92**, 105; L. M. Dorfman and G. E. Adams, Nat. Stand. Ref. Data Ser., Nat. Bur Stand., Washington, 1973, no. 46, p. 1.

It is known [11] that the 'heterolytic' and enzymatic hydroxylation of [4-^{3}H]phenylalanine produce *p*-tyrosine with *ca.* 95% tritium retention. The values for both reactions studied are appreciably lower than this (by

TABLE 4

Effect of superoxide dismutase on total hydroxylation yields

Reaction conditions	Total hydroxylation (%)
(1) 1·56mM-phenylalanine, 0·6mM-Fe^{2+}, 12·8M-tetrahydrobiopterin, oxygen, unbuffered	42·7
(2) As (1) + superoxide dismutase (1 mg cm^{-3})	42·9
(3) As (1) without added Fe^{2+}	16·4
(4) As (3) + superoxide dismutase (1 mg cm^{-3})	24·7
(5) 1·56mM-phenylalanine, 12·8mM-tetrahydrobiopterin in 0·05M-phosphate buffer, pH 7 under air at 40° overnight	10·9
(6) As (5) + superoxide dismutase (1 mg cm^{-3})	12·5

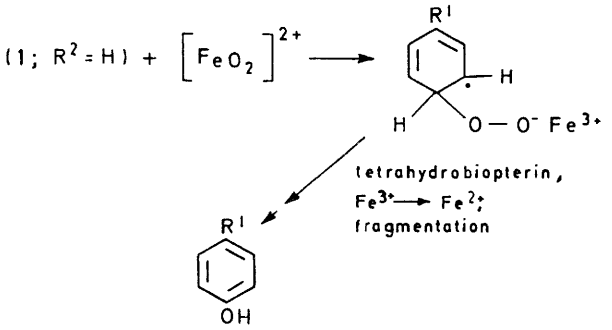
TABLE 5

N.I.H. shift during Fenton reagent- and tetrahydrobiopterin-mediated hydroxylation of [*p*-^{3}H]phenylalanine to *p*-tyrosine

Reaction system *	Retention of tritium in *p*-tyrosine (%) †
Fenton	14 ± 5
Tetrahydrobiopterin	14 ± 5

* Conditions as in Experimental section. † Mean of three determinations ± S.E.M.

about the same factor as observed for other substrate molecules [14]) and are almost identical in these determinations (but this is probably a fortuitous result, the error involved in the estimation being *ca.* 30% of the quoted values). This does indicate however that the reactive



SCHEME 2 Possible monohydroxylation (*e.g. para*) by iron–oxygen complexes

entity in the tetrahydrobiopterin system is free radical in nature.

Conclusions.—The results show that non-enzymatic, tetrahydropterin-mediated, aromatic hydroxylations are extremely complex, and involve free-radical species, though the hydroperoxyl radical chain carrier for tetrahydropterin autoxidation appears to be relatively unreactive toward the aromatic nucleus. It is known [19] that autoxidation of ferrous ion induces aromatic hydroxylation, and in the light of this evidence, iron(II)–oxygen complexes may be implicated both in the presence and absence of reducing agents. The rather lower hydroxylation yield observed in the absence of added ferrous ion is possibly due to the catalytic effect of trace metal impurities in the water and starting materials which cannot be completely removed. The iron–oxygen complexes might well effect free-radical hydroxylation directly (Scheme 2) or might be reduced by tetrahydropterin, leading ultimately to hydroxyl radicals.

EXPERIMENTAL

Liquid scintillation counting for ^{14}C and ^{3}H determination was done with a Nuclear Enterprises NE 8305 counter, using Unisolve 1 Scintillator (Koch–Light). Where samples were burned in order to determine radioactivity, this was done with a Beckman biological material oxidiser. All radiochemicals were purchased from the Radiochemical Centre, Amersham, and used without further purification, and non-radioactive materials used in synthesis and as t.l.c. standards were purchased from Phase Separations, Koch–Light, and B.D.H. Inorganic materials were Analytical Reagent grade. Tetrahydrobiopterin and superoxide dismutase were gifts from Roche Products, and Dr. A. M. Stokes, Oxford University, respectively. Since 2,3- and 2,5-dihydroxyphenylalanines were not commercially available, and were required as t.l.c. standards for product characterisation, they were synthesised by the Erlenmeyer azlactone reaction using the method described by Lambooy.[20] Physical data are given below. M.p.s were determined on a Reichert hot stage and are uncorrected, u.v. spectra were recorded with a Unicam SP700 spectrophotometer, n.m.r. spectra with Perkin-Elmer R14 or Varian HA 100D spectrometer, mass spectra on an A.E.I. MS9 spectrometer, and i.r. spectra on a Perkin-Elmer 157G spectrometer.

2,3-Dihydroxyphenylalanine had m.p. 268—270° (decomp.) [lit., 280 (decomp.),[20] 265° [21]], $\lambda_{max.}$ (pH 1) 279 nm (log ε 3·36) $\nu_{max.}$ (KBr) 3500—2200 (CO_2H) and 1660 (C=O) cm^{-1}, τ (CF_3CO_2H) 2·5br (3H, $\overset{+}{N}H_3$), 3·1 (3H, q, ArH), 5·23br (1H, α-H of alanine), and 6·4 (2H, m, β-H_2 of alanine), *m/e* 197 (5·7%), 179 (2·9), 177 (5·7), 164 (20), 151 (100), 136 (20), 134 (23), 124 (26), 123 (34), 122 (34), 110 (40), 95 (11), 94 (17), 79 (17), 78 (43), 77 (31), 53 (14), 52 (23), 51 (37), and 50 (20) (Found: C, 54·6; H, 5·65; N, 6·95%; M^+, 197·068673. Calc. for $C_9H_{11}NO_4$: C, 54·8; H, 5·6; N, 7·1%; *M*, 197·068802).

2,5-Dihydroxyphenylalanine had m.p. 257·5—258·5° (decomp.) (lit.,[20] 257—258°), $\lambda_{max.}$ (pH 1) 205 (log ε 3·89), 218sh (3·73), and 294 (3·56) nm, $\nu_{max.}$ (KBr) 3545 (N–H), 3500—2300 (CO_2H), and 1630 (C=O) cm^{-1}, τ (CF_3CO_2H) 2·45br (3H, $\overset{+}{N}H_3$), 2·90 (1H, ArH), 3·08 (2H, ArH), and 5·25br (1H) and 6·45 (2H, m) (α-H and β-H_2 of alanine), and appears to lose elements of water on heating to give M^+ 179·058694 (calc. for $C_9H_9NO_3$: *M*, 179·058238) (Found:

[19] M. B. Dearden, C. R. E. Jefcoate, and J. R. Lindsay Smith, Amer. Chem. Soc. Advances in Chemistry Series No. 77, 1968, p. 260.

[20] J. P. Lambooy, *J. Amer. Chem. Soc.*, 1954, **76**, 133.

[21] G. R. Clemo and F. K. Duxbury, *J. Chem. Soc.*, 1950, 1795.

C, 50·5; H, 6·0; N, 6·5. Calc. for $C_9H_{11}NO_4,H_2O$: C, 50·2; H, 6·1; N, 6·5%) (ref. 22 reports this compound as monohydrate).

Hydroxylation Reactions.—Solutions of ferrous ammonium sulphate, EDTA (where present), and phenylalanine (containing *ca.* 4 μCi cm^{-3} of [^{14}C-*carboxy*]phenylalanine) were mixed to give the concentrations shown in the Results section (Table 2) in a final volume of 1 cm^3, and the temperature was adjusted to 40° by immersion in the water-bath. Catalase and superoxide dismutase, where used, were contained in the phenylalanine solution at the appropriate concentration. For the Fenton reactions hydrogen peroxide solution (100 μl) at the appropriate concentration (standardised by the iodide method) was added to give the concentrations of Table 2, and for the tetrahydrobiopterin reactions the correct weight of this compound was introduced as a freshly made solution in water (100 μl). The former was incubated for 3 h in equilibrium with air, and the latter for 3 h with the passage of a steady stream of oxygen gas.

Yield Determinations.—After incubation, the solutions (10 μl) were applied to a cellulose layer (MN300; 0·1 mm; Polygram foil without fluorescent indicator) alongside appropriate authentic materials as standards, and eluted with solvent system (1) of Table 1. The foils were dried, marked with radioactive materials at appropriate points not coinciding with chromatograms, and exposed to Ilford *X*-ray film in the dark for 3—7 days, and the autoradiograms were developed. At this point, non-radioactive standards were located with ninhydrin spray, after marking off the product chromatograms (to avoid loss of CO_2H and, therefore, label). Bands corresponding to reaction products and unchanged phenylalanine were located by aligning the radioactive markers on the plate and autoradiogram; each was carefully removed by sectioning the plate, and extracted overnight with a known volume of aqueous 4% ammonia (10 cm^3). Extracts (5 cm^3) were thoroughly mixed with scintillator (10 cm^3) and counted. Background count was obtained from a mixture of aqueous 4% ammonia (5 cm^3) and scintillator (10 cm^3). Total recovery in all cases was found to be *ca.* 95% by comparison with count rates of unchromatographed reactant solutions (10 μl in 5 cm^3 4% ammonia, plus scintillator), and yields are based on total recovered radioactivity.

Tritium Retention of p-Tyrosine. N.I.H. Shifts.—The incubation procedure was as above except that [^{14}C]phenylalanine was replaced by *para*-tritiated compound (0·2 mCi cm^{-3}). Concentrations were as in C and F of Table 2. After incubation each reaction mixture was introduced into boiling water saturated with an accurately known weight (1·5—2·0 g) of non-radioactive *p*-tyrosine, and the latter crystallised out on cooling. It was then recrystallised from water eight times and a known weight finally purified by t.l.c. on cellulose layer (with fluorescent indicator). The *p*-tyrosine band was located as absorbing material under u.v. light (254 nm), removed, and extracted into 2M-hydrochloric acid (2 cm^3). This solution could not be counted directly owing to interference from the fluorescent indicator which appeared to be extracted into the hydrochloric acid Therefore, samples (100 μl) were passed through the biological material oxidiser in the usual way, and the tritiated water was collected in a dry ice–acetone trap, taken up with scintillator (10 cm^3), and counted In this way the *apparent* yield of *p*-tyrosine could be computed and the tritium retention values (%) were obtained by comparison with the ^{14}C experiments On passing unchanged phenylalanine through the above procedure radioactive *p*-tyrosine was found at an impurity level of 0·295% This could not be removed without risk of contamination by other materials (*e.g.* from chromatography procedures) which might upset the reactions and significantly alter yields. Therefore, all N.I.H. shift values were corrected for this, even though tyrosine might also react to some extent. (No tritium is detectable in a position other than *para* in the starting material, thereby ruling out errors due to non-specificity of labelling.[23])

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[22] J. P. Lambooy, *J. Amer. Chem. Soc.*, 1949, **71**, 3758.
[23] Radiochemical Centre, Amersham, personal communication.