Studies of aromatic hydroxylation

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STUDIES OF AROMATIC HYDROXYLATION

by

WILLIAM ROY GRETTON, B.Sc.

A Doctoral Thesis

submitted in partial fulfilment of the requirements

for the award of

Doctor of Philosophy of the Loughborough University of Technology

December 1973

Supervisors: Professor G.W. Kirby, M.A., Ph.D., Sc.D., F.R.I.C.
W.R. Bowman, B.Sc., Ph.D.

Department of Chemistry

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To

MARY

and

HANNAH
SUMMARY

The conversion of $[4^{-3}\text{H}]$- and $[4^{-3}\text{H}; 3,5^{-2}\text{H}_2]$-phenylalanine into tyrosine by cultures of *Pseudomonas* sp. (NCIB 9289) has been studied. From a consideration of the degree of retention of tritium observed during the hydroxylation of the two precursors a kinetic isotope effect, $k_D/k_T = 2.8 \pm 0.1$ ($k_H/k_D = 10 \pm 1$), has been calculated for the final aromatisation process following the NIH shift. The relevance of this result to the overall reaction pathway is discussed in the light of similar data derived from possible chemical models for the biological process.

The incorporation of $[3^{-3}\text{H}]$, $[4^{-3}\text{H}]$, and $[4^{-3}\text{H}; 3,5^{-2}\text{H}_2]$-labelled specimens of phenylalanine and cinnamic acid into norpluviine and haemanthamine in "Texas" daffodils has been studied. The degree of retention of tritium in the alkaloids derived from a particular phenylalanine precursor was very similar to that obtained using a cinnamic acid precursor bearing an identical isotopic labelling pattern. From a consideration of the tritium retentions in the alkaloids derived from the para-tritiated precursors a kinetic isotope effect, $k_H/k_T = 19 \pm 5$ ($k_H/k_D = 7.7 \pm 1.4$), has been calculated for the final aromatisation process following the NIH shift which accompanies the first hydroxylation of the aromatic ring.

The thermolysis of benz[c,d]indazole 1,2-dioxide in $[4^{-3}\text{H}; \text{methyl-}^{14}\text{C}]$-toluene has been investigated. Toluene derivatives resulting from nuclear hydroxylation, and
from oxidation of the side-chain, were isolated, and the degree of retention of tritium was measured in each case. The results are compared with data derived from experiments with other chemical oxidants, and with mono-oxygenase systems.
ACKNOWLEDGEMENTS

I would like to express my gratitude to Professor Kirby and Dr. Bowman for their friendly guidance and encouragement throughout the course of this work.

I am indebted to the Science Research Council for financial support, and to Dr. N. Johns and Dr. B. C. Webster for assistance as indicated in the text.

I am grateful to Mrs. C. Bartrop and her coworker for their valuable contributions towards the typing of this manuscript.

Finally my thanks are due to my wife for proof reading and duplicating these pages, and for her encouragement and understanding during the past three years.
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PART I

Hydroxylation-Induced Migration of Isotopic Hydrogen in vivo
INTRODUCTION

The 1,2-shift of a ring substituent, which can accompany enzymic hydroxylation of an aromatic substrate, has been observed in a wide range of biological systems. Early studies of this phenomenon, which became known as the "NIH shift", were carried out in the National Institutes of Health (Maryland, U.S.A.), and involved systems in which the hydroxyl group was introduced para to the principal substituent of the ring. More recently, migration has been shown to accompany ortho and meta hydroxylations. The survey that follows, while not intended to be exhaustive, illustrates the widespread occurrence of the NIH shift accompanying biological aromatic hydroxylation.

Migration accompanying para hydroxylation.

Instances of tritium and deuterium migration in simple benzenoid compounds are summarised in Tables 1 and 2 respectively. The hydroxylation of [4-3H]phenylalanine is a classic example, as attempts to devise an assay for the enzyme using this substrate led to the discovery of the NIH shift.\(^1\)

Migration of substituents other than hydrogen isotopes has been observed. A 1,2-shift of chlorine accompanying the hydroxylation of 2,4-dichlorophenoxyacetic acid (I) in Aspergillus niger has been reported.\(^{16}\) A similar phenomenon has been observed in the stem tissue of Phaseolus vulgaris.\(^{17}\) The thiazole acetic acid derivative (II) is metabolised in mammals to a mixture of phenolic products in which the chlorine either migrates or is eliminated.\(^{18}\) Further investigations in the National Institutes of Health have shown that bacterial phenylalanine hydroxylase will catalyse the conversion of 1.
### TABLE 1

Examples of tritium migration induced by para hydroxylation of simple benzenoid substrates

![Diagram showing migration of tritium from one position to another in a benzenoid substrate.]

<table>
<thead>
<tr>
<th>Nature of R</th>
<th>Hydroxylation system</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>-CH₂-CH₂-NH₂</td>
<td>Liver phenylalanine 4-hydroxylase</td>
<td>2,3</td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas</em> &quot; &quot; &quot; &quot;</td>
<td>2,3</td>
</tr>
<tr>
<td></td>
<td>Barley <em>in vivo</em></td>
<td>4</td>
</tr>
<tr>
<td>-NH₂COCH₃</td>
<td>Adrenal tyrosine hydroxylase</td>
<td>5</td>
</tr>
<tr>
<td>-CH₂-CH₂-NH₂</td>
<td>Liver microsomal aryl hydroxylase</td>
<td>6,7</td>
</tr>
<tr>
<td></td>
<td>&quot; &quot; &quot; &quot;</td>
<td>7</td>
</tr>
<tr>
<td>-NH₂</td>
<td><em>Pisum sativum in vivo</em></td>
<td>8</td>
</tr>
<tr>
<td>-CH=CH₂CO₂H</td>
<td>&quot; &quot; cinnamic acid 4-&quot; hydroxylase</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Buckwheat <em>in vivo</em></td>
<td>10</td>
</tr>
<tr>
<td></td>
<td><em>Catalpa hybrida in vivo</em></td>
<td>11</td>
</tr>
</tbody>
</table>

2.
### Table 2

Examples of deuterium migration induced by para hydroxylation of simple benzenoid substrates

![Chemical Structure](image)

<table>
<thead>
<tr>
<th>Nature of R</th>
<th>Hydoxylating system</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>-NHCOCH$_3$</td>
<td>Liver microsomal aryl hydroxylase</td>
<td>6,12,13</td>
</tr>
<tr>
<td>-NH$_2$, -NHMeCOCH$_3$</td>
<td>&quot; &quot; &quot; &quot;</td>
<td>14</td>
</tr>
<tr>
<td>-NHCONH$_2$, -NHCOH, -OPh, -Ph, -NHCOPh, -F, -Cl, -Br</td>
<td>&quot; &quot; &quot; &quot;</td>
<td>12,14</td>
</tr>
<tr>
<td>-OCH$_3$, -CH$_3$</td>
<td>Aspergillus niger</td>
<td>15</td>
</tr>
<tr>
<td>-NO$_2$, -CN, -CONH$_2$</td>
<td>Rodent <em>in vivo</em></td>
<td>14</td>
</tr>
<tr>
<td>-NH$_2$</td>
<td>Pseudomonas phenylalanine hydroxylase</td>
<td>1</td>
</tr>
<tr>
<td>-CH$_2$-CH$_2$COOH</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

3.
4-chlorophenylalanine to 3-chloro-4-hydroxyphenylalanine,\textsuperscript{19} of 4-bromophenylalanine to 3-bromo-4-hydroxyphenylalanine,\textsuperscript{19} and of 4-methylphenylalanine to 3-methyl-4-hydroxyphenylalanine.\textsuperscript{20} The latter case is the only reported example of simple alkyl migration during enzymic aryl hydroxylation.

\textbf{Migration accompanying ortho hydroxylation.}

When \([2-^{3}\text{H}]\text{cinnamic acid}\) was administered to young Gaultheria tissue,\textsuperscript{21} the \underline{ortho-coumaric acid}, coumarin, and salicylic acid derived therefrom contained tritium \textit{ortho} to the position of hydroxylation (Scheme 1). Daly and Jerina\textsuperscript{22} have demonstrated that when toluene, anisole, and acetanilide, each deuteriated in the \textit{ortho} position, undergo hydroxylation in the presence of hepatic microsomes, one product in each case is the \textit{ortho}-hydroxy derivative, the deuterium having migrated to the \textit{meta} position. The retention of deuterium during \textit{ortho} hydroxylation was of a similar magnitude to that observed during hydroxylation \textit{para} to the same substituent, in spite of the fact that in the former case there was only one ring position to which the isotope could migrate. Similar experiments with \textit{Aspergillus niger}\textsuperscript{15} have revealed that \textit{ortho} hydroxylation of deuteriated toluene, anisole, and phenylacetic acid in this organism is accompanied by a \(1,2\)-shift of deuterium from the position of hydroxylation.

\textbf{Migration accompanying meta hydroxylation.}

An NIH shift of tritium accompanying the \textit{meta} hydroxylation of a phenylalanine unit has been reported. When \([3-^{3}\text{H}]\text{phenylalanine}\) was administered to cultures of \textit{Penicillium cyclopium} Westling the resulting cyclopenol (III) retained ca. 65\% of the tritium.\textsuperscript{23} A further example is the microsomal hydroxylation.
The percentages refer to the retention of isotope accompanying hydroxylation at an ortho position bearing tritium, and have been arrived at by doubling the observed percentage loss of isotope in the formation of the product, and subtracting this figure from 100%. 

5.
of [3⁻²H]chlorobenzene, which gives a meta hydroxylated product in which a 24% migration and retention of the deuterium is observed.  

**Hydroxylation-induced migration in compounds with fused aromatic rings.**

Renson et al. 24 found that when [5⁻³H]tryptophan (IV) was incubated in the presence of tryptophan 5-hydroxylase from mouse mast cells the 5-hydroxytryptophan produced contained at least 85% of the original tritium, the latter having migrated to C-4. Hydroxylation of either [1⁻²H]- or [2⁻²H]naphthalene in the presence of hepatic microsomes at pH 8 leads to the formation of [2⁻²H]naphth-1-ol (V) in which 68% of the deuterium is retained. 25, 26 The latter value is subject to error limits of ±8%, since [4⁻²H]-, [5⁻²H]-, and [8⁻²H]-naphth-1-ol are also formed, and the overall retention of deuterium (not quoted in the literature) was measured to an accuracy of ±2%. A 13% retention of deuterium was observed when [6⁻²H]naphthyl-2-oxyacetic acid was hydroxylated at C-6 in Aspergillus niger. 15

**Mechanisms for the NIH shift.**

To acquire a thorough knowledge of enzymic aromatic hydroxylation it will be necessary to understand (i) the nature of the oxygenating species, (ii) the nature of the oxygenated intermediates involved, (iii) the mechanism of the 1,2-migration, (iv) the extent of enzymic involvement at each step of the hydroxylation process, and (v) the relationship between the structure of the substrate and the relative importance of the possible reaction pathways.

An acceptable mechanism for the NIH shift must be able to account satisfactorily for the observed degree of retention of
the migrating species, which can vary according to the substrate and, in some cases, according to the conditions. A number of examples are presented in table 3.

It can firstly be stated that no kinetic isotope effect is observed when the hydrogen atom at the position of hydroxylation is replaced by deuterium or tritium; in other words, protiated, deuteriated, and tritiated species are metabolised at equal rates. This has been carefully established with phenylalanine\(^3\) and acetanilide.\(^{27}\) Secondly, the 1,2-shift of a substituent is not a general feature of electrophilic aromatic substitution,\(^{28}\) although it has been shown to occur when aromatic rings are oxygenated by certain non-enzymic oxygen-donors; the significance of these reactions will be discussed later.

Shortly after the discovery of the NIH shift a mechanism for aromatic hydroxylation was proposed\(^{24}\) which involved attack by HO\(^+\) (or its equivalent in an enzyme-oxygen complex) to form the cationic species (VI) (Scheme 2), where X might be \(^2\)H, \(^3\)H, Cl or Br. (VI) could then rearrange to (VII) via path a, the driving force for the migration of X being the greater delocalisation of the positive charge in (VII) by involvement of a lone pair on oxygen. Loss of H\(^+\) or X\(^+\) from (VII) and re-aromatisation would lead to the phenolic products (VIII) or (IX) respectively. Alternatively, loss of X\(^+\) might occur without migration (path b), resulting in the formation of product (IX). According to this hypothesis, for a given substrate the relative amounts of the two products would depend upon two factors: (i) the tendency of (VI) to aromatise by loss of X\(^+\) without migration, and (III) assuming that an appreciable amount of (VII) is formed, the relative strengths
### TABLE 3

**Extent of retention of the migrating species during para hydroxylation**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Hydroxylating system</th>
<th>Migrating species</th>
<th>% retention</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylalanine</td>
<td>Pseudomonas or liver phenylalanine 4-hydroxylase</td>
<td>$^3\text{H}$</td>
<td>87-94</td>
<td>3</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>$^2\text{H}$</td>
<td>ca. 70</td>
<td>1</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>Hepatic aryl hydroxylase</td>
<td>$^3\text{H}$</td>
<td>91</td>
<td>7</td>
</tr>
<tr>
<td>Acetanilide</td>
<td>&quot;</td>
<td>$^3\text{H}$</td>
<td>pH 7; 63</td>
<td>6</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>$^2\text{H}$</td>
<td>pH 8.5; 45</td>
<td>6</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>$^2\text{H}$</td>
<td>pH 9.2; 36</td>
<td>6</td>
</tr>
<tr>
<td>Aniline</td>
<td>&quot;</td>
<td>$^3\text{H}$</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>$^2\text{H}$</td>
<td>6</td>
<td>14</td>
</tr>
</tbody>
</table>
of the C-H and C-X bonds. In the case of hydrogen isotopes, bond strengths decrease in the order $C^3\text{H} > C^2\text{H} > C^1\text{H}$. Hence the high degree of tritium retention observed when $[4^3\text{H}]$phenylalanine was enzymically converted to tyrosine could be readily explained, assuming a high degree of migration of tritium.

This hypothesis was reasonably successful in accounting for the fact that substrates in which the positive charge on the intermediate (VI) could be delocalised by electron donation from the side chain (e.g. acetanilide, Scheme 3) exhibited a lower value for migration and retention than substrates which could not stabilise the positive charge in this way (e.g. phenylalanine and amphetamine).

The operation of a "direct loss" pathway is clearly illustrated in the para hydroxylation of deuteriated chlorobenzene\(^{14,22}\) where the degree of retention of isotope is considerably greater when deuterium is originally present in the meta position, than when it migrates from the para position (Scheme 4). This is true even when allowance is made for the two alternative directions of migration available for the species originally at the para position. On the other hand, a "direct loss" pathway does not appear to be operative\(^{25}\) in the hydroxylation of $[1^{-2}\text{H}]$- and $[2^{-2}\text{H}]$naphthalene (\textit{vide supra}).

An alternative mechanism was considered, involving the addition of oxygen, migration of the para substituent, and removal of an adjacent proton by the enzyme in a concerted fashion (Scheme 5). Convincing evidence against this hypothesis was obtained in studies of the hydroxylation of $[3,5^{-3}\text{H}_2]$phenylalanine, where a concerted process would have led to complete elimination of one of the

11.
SCHEME 4

rodent in vivo

Percentages refer to retention of deuterium in the product

SCHEME 5

SCHEME 6

Percentages refer to retention of deuterium in the product
tritium atoms, equivalent to half of the total radioactivity.

In fact, less than 5% of the activity was lost. A similar result was obtained with [3,5-3H]acetanilide. Hence it was concluded that the enzyme played no role in the hydroxylation beyond the addition of oxygen to a specific position on the molecule.

Subsequent to the formulation of these ideas, further investigations have led to a modified concept of the nature of the oxygenating species, and a more elaborate view of the intermediates involved in the hydroxylation process. The possibility that an arene oxide might be the initial intermediate in aromatic hydroxylation has been realised for some time, but recently the National Institutes of Health group have conducted convincing experiments which implicate the involvement of arene oxides in the oxidative metabolism of aromatic substrates. [4-2H]3,4-Epoxy-3,4-dihydrotoluene was synthesised, and the retention of deuterium upon rearrangement to p-cresol, the sole product, corresponded closely to that observed after enzymic para-hydroxylation of [4-2H]toluene (Scheme 6). 1,2-Epoxy-1,2-dihydronaphthalene has been isolated from an incubation of naphthalene with hepatic microsomes; this fact, considered in conjunction with the observation that the 1,2-oxide, deuteriated at C-1, rearranges to 1-naphthol with retention of 80% of the deuterium at C-2, demonstrates conclusively that the oxide is an intermediate in the hydroxylation. Photolysis of pyridine N-oxide in the presence of para-deuteriated anisole, chlorobenzene, bromobenzene, acetanilide, and toluene, using dichloromethane as solvent, gave para-hydroxylated products in which the percentages of deuterium
retained were similar to those resulting from the microsomal hydroxylation of the same substrates.\textsuperscript{34} Furthermore, 1,2-epoxy-1,2-dihydronaphthalene was one of the products formed when naphthalene was oxidised by the same reagent.

Other workers have shown that microsomal metabolism of the polycyclic hydrocarbons phenanthrene, benz[a]anthracene, and dibenz[a,h]anthracene, involves formation of the oxides (IX), (X), and (XI) respectively.\textsuperscript{35,36}

Conclusive proof that arene oxides are obligatory intermediates in the hydroxylation of substrates such as phenylalanine may be difficult to obtain. Investigations into the properties of methyl-substituted arene oxides have shown that their stability is markedly increased by alkyl substitution of the oxirane ring, and decreased by alkyl substitution at other ring positions.\textsuperscript{37} Hence enzymic oxygenation of aromatic compounds leading to para-substituted phenols would generate very unstable arene oxides, not well suited to isolation and identification.

The nature of the products arising from the spontaneous isomerisation of a variety of methyl-substituted arene oxides is compatible with the intermediacy of these oxides in the microsomal metabolism of the parent hydrocarbons.\textsuperscript{37} The direction of opening of the oxirane ring is strongly influenced by the alkyl side-chain. For instance, 3,4-epoxy-3,4-dihydrotoluene rearranges exclusively to \(\beta\)-cresol, while 2,3-epoxy-2,3-dihydrotoluene rearranges exclusively to \(\alpha\)-cresol (Scheme 7) under the influence of the inductive (or hyperconjugative) effect of the methyl substituent.\textsuperscript{37} Significantly, no \(m\)-cresol is formed during microsomal hydroxylation of toluene.
SCHEME 7

\[
\text{(canonical form stabilised by Me)}
\]

\[
\text{(no additional stabilisation by Me)}
\]

SCHEME 8

16.
Ortho hydroxylation of a mono-substituted benzene ring might occur via formation of a 1,2-oxide, or a 2,3-oxide. Where the hydroxylation is accompanied by a high degree of migration of an ortho label to the 3-position (e.g. Scheme 1) intermediacy of a 1,2-oxide can be discounted, unless a variation of the generally-accepted mechanism for the NIH shift is envisaged, in the form of a 1,2-migration of the isotope away from, rather than across, the oxirane ring (Scheme 8). Steric factors are believed to militate strongly against the formation of such 1,2-oxides in microsomal hydroxylations; however, formation of the 1,2-oxides of phenoxyacetic acid, phenylacetic acid, and toluene has been invoked as a possible explanation for the low degree of retention of isotope when the respective ortho-deuteriated substrates are metabolised in fungi. Isomerisation of the 1,2-oxide might result in migration of the deuterium to the 1-position, but pre-aromatisation would ensure elimination of the label.

Examples are known where aryl hydroxylation of a compound with an unsymmetrically substituted benzene ring leads to migration of isotopic hydrogen to alternate positions on the ring, as occurs during metabolism of [3-2H]3-deoxyoestrone in rabbits (Scheme 9). The intermediacy of two different arene oxides has been postulated to account for this type of behaviour; migration to C-2 would implicate the involvement of the 2,3-oxide, while migration to C-4 would implicate the 3,4-oxide. Here again, an alternative explanation based upon the type of mechanism illustrated in Scheme 8 cannot be ruled out at the present.

Concurrent with the implication of arene oxides as intermediates in aromatic oxygenations there has been a reconsideration
SCHEME 9

(81% retention of $^2$H)

(50% retention of $^2$H)

(20% retention of $^2$H)
of the possible nature of the active oxygen species associated with mono-oxygenases. An "oxenoid" mechanism, involving oxygen atom transfer rather than attack by HO$^+$ is favoured. In this context the oxygen atom is referred to as "oxene" because it is isoelectronic with the reactive intermediates carbene and nitrene, and is thought to behave in an analogous manner in a number of reactions. For example, carbenes in a singlet spin-state add in a concerted fashion to benzene to give derivatives of norcaradiene, the carbon analogue of benzene oxide (Scheme 10). Ring expansion produces the isomeric cycloheptatriene, which is analogous to oxepin, the isomer of benzene oxide. Extending the analogy, it would be reasonable to expect oxene to undergo addition to olefinic double bonds to give epoxides, and to insert into carbon-hydrogen bonds to give alcohols or phenols. The latter might arise from triplet oxene without the intermediacy of an arene oxide. Metabolites of these types are indeed typical products of mono-oxygenase activity. The precise nature of the enzyme-activated oxygen donor in these reactions remains a matter for conjecture. It has been proposed that a peroxide formed by addition of molecular oxygen to the reduced cofactor may be involved, but an oxo-iron species is preferred.

Scheme 11 shows a number of pathways for aromatic hydroxylation via an arene oxide. To simplify the diagram, steps involving re-aromatization by direct loss of hydrogen isotope from the para position have not been included.

In their studies of the aromatisation of 1,2-epoxy-1,2-dihydrobenzene in aqueous solutions, Kasperek and Bruice
SCHEME 10

\[
\text{benzene} + \text{carbene} \rightarrow \text{benzene derivative with R}_{1} \text{ and R}_{2}
\]

"oxene"

\[
\text{benzene} + \text{oxene} \rightarrow \text{benzene derivative with oxygen atom} \quad \leftrightarrow \text{cyclobutadiene}
\]
Scheme II

H' and H'' are dissimilar isotopes of hydrogen.
discovered that the rate-constant-pH profile for the rearrangement to phenol revealed the operation of acid catalysis below pH6, and a pH-independent region above pH6. This observation lends support to the view that arene oxides, if formed as a result of hydroxylase activity under physiological conditions, would rearrange to phenols without acid catalysis.

Assuming that no loss of hydrogen isotope occurs without migration, the degree of retention of the isotope will depend upon the relative ease of rupture of the carbon-hydrogen isotope bonds in species such as (XII) or (XIII). This hypothesis finds support in data from the majority of experiments where deuterium and tritium labelling of the aromatic ring have been employed. However, an apparent exception was reported by Bowman, Bruce, and Kirby, after studying the incorporation of $[\beta^{-1}^{4}\text{C}; 4^{-3}\text{H}]\text{cinnamic acid and } [\beta^{-1}^{4}\text{C}; 4^{-3}\text{H}; 3,5^{-2}\text{H}_2]\text{cinnamic acid into the alkaloids capsaicin (XIV) and norpluviine (XV) of Capsicum annuum and "Texas" daffodils, respectively. On the basis of the mechanism proposed in Scheme 10 the loss of tritium after the migration induced by cinnamate hydroxylation would occur from intermediates such as (XVI) or (XVII). The degree of tritium loss would, to a first approximation, be governed in (XVI) by the value of the primary kinetic isotope effect $k_H/k_T$ for the loss of isotope accompanying enolisation, and in (XVII) by the smaller quantity $k_D/k_T$. However, in both cases retention of ca. 50% of the tritium was observed, suggesting that the species originally present at the meta position had been stereospecifically removed, presumably by enzymic action; i.e. at the first hydroxylation of the ring, all the migrating
tritium was retained in the molecule, half of this being eliminated at the second hydroxylation. This conclusion was supported when it was shown that $[^\beta-^{14}\text{C};\, ^3\text{H}]$cinnamic acid was incorporated into norpluviine with a 72% loss of tritium, rather than the slightly under 50% loss predicted by the operation of an $^3\text{H}/^\text{T}$ isotope effect. It was suggested that the apparent conflict between these and previous observations could be resolved by assuming that two enzymes were involved in the aryl hydroxylase system of an intact organism: an oxygenase, giving rise to an arene oxide, and an isomerase, converting the arene oxide to a phenol with complete retention of the migrating hydrogen, irrespective of its isotopic nature. The isomerase was assumed to be labile, and readily lost when purification of the hydroxylase system was attempted.

The present work was initiated with the intention of testing this hypothesis in the case of the phenylalanine hydroxylase system of a *Pseudomonas* species. In addition, the incorporation of ring-labelled aromatic precursors into Amaryllidaceae alkaloids has been investigated further.
DISCUSSION

Section A. Hydroxylation of Phenylalanine by Pseudomonas species

Our objectives in studying the hydroxylation of phenylalanine in vivo were, firstly, to seek further evidence for the existence of an arene oxide isomerase, as proposed by Bowman et al.,\textsuperscript{42} and secondly, in the absence of such evidence, to attempt to measure the kinetic hydrogen isotope effect associated with the enolisation of an intermediate such as (XVIII) or (XIX), where H' and H" represent dissimilar isotopes of hydrogen, following the NIH shift.

The phenylalanine hydroxylase system of \textit{Pseudomonas} sp. (NCIB 9289, ATCC 11299a) has been studied extensively by Guroff and his co-workers\textsuperscript{43-46}, and there was much to commend its selection for our investigations. When the bacterium is cultured in a medium containing L-phenylalanine as the sole carbon source, tyrosine appears as the most abundant amino acid in the medium. The crude enzyme system lends itself to progressive purification, a 30-fold increase in activity having been achieved\textsuperscript{45}. It was also known\textsuperscript{2,3} that conversion of \(\left[4^{-3}\text{H}\right]\)phenylalanine into tyrosine by purified hydroxylase preparations from \textit{Pseudomonas} was accompanied by migration and retention of more than 90% of the tritium.

One might attempt to measure the kinetic isotope effect \(k_H/k_T\) by using \(\left[4^{-3}\text{H}\right]\)phenylalanine as substrate, but the value obtained would be extremely sensitive to experi-
mental errors. For instance, an overall tritium retention of 95% would correspond to a $k_H/k_T$ of 19, assuming that no loss of tritium occurs without migration (cf. path b in Scheme 2). If, however, we assume for the sake of argument that there is a 2% loss of tritium without migration, a 95% retention would correspond to a $k_H/k_T$ of ca. $\frac{27}{3} = 32$. Even if non-migration loss was negligible an experimental value of 95±2% for tritium retention would mean that $k_H/k_T$ might lie at any point between 13 and 32.

Use of deuterium in the place of tritium should reduce the margin of error. For instance, retentions of 85% and 90% of the isotope accompanying hydroxylation of $[4\cdot{}^2H]$phenylalanine would correspond to values for $k_H/k_D$ of 5 and 9 respectively. Loss of deuterium without migration might, however, affect the accuracy of the measurement considerably. Furthermore, measurement of the deuterium content of the derived tyrosine would require isolation of the amino acid without substantial dilution with unlabelled material, a condition which is usually difficult to satisfy with intact organisms. A preferable approach to the problem can be made by using $[4\cdot{}^3H;3\cdot{}^2H_2]$phenylalanine (XX) as precursor, which would permit measurement of the isotope effect $k_D/k_T$, a smaller quantity than $k_H/k_D$. The value of the latter could then be deduced by use of the Swain relationship, $k_H/k_T = (k_H/k_D)^{1.442}$, assuming that secondary isotope effects are negligible. (The latter point will be discussed later).
Our approach was to study the hydroxylation of DL-[4-\textsuperscript{3}H]- and DL-[4-\textsuperscript{3}H;3,5-\textsuperscript{2}H\textsubscript{2}]-phenylalanine in parallel cultures of \textit{Pseudomonas}. The importance of feeding the non-deuteriated precursor lay in the need to demonstrate that non-migration loss of tritium does not occur to an extent which would significantly affect the value of $k_D/k_T$ obtained from the retention of tritium in the product from the dideuteriated precursor.

Labelled specimens of phenylalanine were prepared as follows. 4-Iodotoluene was treated successively with lithium metal and tritiated water to produce [4-\textsuperscript{3}H]toluene which was photobrominated to give [4-\textsuperscript{3}H]benzyl bromide. Condensation of the latter with 3-phenylhydantoin in the presence of magnesium methyl carbonate\textsuperscript{50} yielded [4-\textsuperscript{3}H]-benzyl-3-phenylhydantoin\textsuperscript{51}, which on hydrolysis with aqueous barium hydroxide\textsuperscript{52} gave DL-[4-\textsuperscript{3}H]phenylalanine. 4-Aminotoluene hydrochloride was converted\textsuperscript{53}, by repeated exchanged in deuterium oxide, into 4-amo-[3,5-\textsuperscript{2}H\textsubscript{2}]toluene. Diazotisation and treatment with potassium iodide gave 4-iodo-[3,5-\textsuperscript{2}H\textsubscript{2}]toluene which was converted, via [4-\textsuperscript{3}H; 3,5-\textsuperscript{2}H\textsubscript{2}]toluene, into DL-[4-\textsuperscript{3}H;3,5-\textsuperscript{2}H\textsubscript{2}]-phenylalanine (Scheme 12). The molecular ion regions of the mass spectra of dideuteriated and non-deuteriated phenylalanine are depicted in Fig.1.

The position of tritium in both precursors was confirmed by degradation. Treatment of the phenylalanine with alkali and dimethyl sulphate gave cinnamic acid, which was oxidised to benzoic acid. Treatment with hydrazoic acid gave aniline, which was acetylated and brominated to yield...
SCHEME 12

Chemical reactions and structural formulas are shown with specific reagents and conditions indicated in the diagram. The reactions involve aromatic compounds and involve transformations such as deuterium exchange, bromination, and reduction with lithium. The final product is shown with specific substituents and configurations.
FIG. 1

Mass spectrum of dideuteriated phenylalanine

% M⁺

Mass spectrum of non-deuteriated phenylalanine

% M⁺
4-bromoacetanilide. The labelled benzoic acids were converted into their 3,5-dinitroderivatives. The results of these degradations are summarised in Table 4.

The tritiated phenylalanines were each mixed with DL-[carboxy-\(^{14}C\)]phenylalanine to provide samples of known \(\text{^{3}H}/\text{^{14}C}\) ratio, and administered to \textit{Pseudomonas} cultures growing in a medium containing non-radioactive L-phenylalanine and mineral salts. The course of the conversion into tyrosine was followed by radioactive scanning of paper chromatograms of the culture medium. Radioactive tyrosine was detectable after ca. 0.5 h., and became the major radioactive constituent after ca. 5 h. (Fig. 2). When an adequate conversion had occurred non-radioactive L-tyrosine was added, in dilute hydrochloric acid, to the medium and the cells were removed by centrifugation. The supernatant was adjusted to pH 6 with aqueous sodium hydroxide and the precipitated tyrosine was recrystallised to constant specific radioactivity and \(\text{^{3}H}/\text{^{14}C}\) ratio. Repeated crystallisation of the purified tyrosine did not cause loss of tritium by exchange. The experiment was carried out near pH 6, conditions which were not expected to cause loss of tritium from positions \textit{ortho} to the phenolic hydroxyl group.

In order to establish that "scrambling" of the radiolabels had not occurred during metabolism by \textit{Pseudomonas}, degradations were carried out on the tyrosine specimens. Treatment with alkali and dimethyl sulphate yielded 4-methoxycinnamic acid without loss of \(\text{^{3}H}\) or \(\text{^{14}C}\). The latter derivative was oxidised to 4-methoxybenzoic acid by treatment with alkaline permanganate, resulting
### TABLE 4

Degradation of cinnamic acids derived from DL-[4-3H]- and DL-[4-3H; 3,5-2H2]-phenylalanine

<table>
<thead>
<tr>
<th>Labelling pattern</th>
<th>Benzoic acid</th>
<th>3,5-Dinitrobenzoic acid</th>
<th>Acetanilide</th>
<th>4-Bromoacetanilide</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-3H</td>
<td>1.02</td>
<td>1.00</td>
<td>1.00</td>
<td>0.01</td>
</tr>
<tr>
<td>4-3H; 3,5-2H2</td>
<td>0.99</td>
<td>0.99</td>
<td>1.00</td>
<td>0.01</td>
</tr>
</tbody>
</table>

### TABLE 5

Incorporation of DL-phenylalanine into L-tyrosine by Pseudomonas

<table>
<thead>
<tr>
<th>Labelling pattern</th>
<th>Precursor</th>
<th>Tyrosine</th>
<th>4-Methoxy-cinnamic acid</th>
<th>Incorporation (%)</th>
<th>3H retention (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-3H; carboxy-14C</td>
<td>5.43</td>
<td>5.20</td>
<td>5.20</td>
<td>16.5</td>
<td>95.8</td>
</tr>
<tr>
<td>ditto</td>
<td>4.23</td>
<td>3.99</td>
<td>3.96</td>
<td>6.2</td>
<td>94.0</td>
</tr>
<tr>
<td>4-3H; 3,5-2H2; carboxy-14C</td>
<td>5.88</td>
<td>4.38</td>
<td>4.45</td>
<td>18.2</td>
<td>75.1</td>
</tr>
<tr>
<td>ditto</td>
<td>5.01</td>
<td>3.70</td>
<td>3.67</td>
<td>6.0</td>
<td>73.6</td>
</tr>
</tbody>
</table>

For further details see Table 9 (Experimental section).
FIG. 2

Scanning of radioactive chromatograms from _Pseudomonas_ medium

Time elapsed after addition of radioactive phenylalanine

- 0.5 h
- 3.0 h
- 4.5 h
- 5.5 h
- 6.5 h

phenylalanine  tyrosine  baseline
in >98% removal of $^{14}$C, and complete retention of $^3$H. Treatment of the tyrosine with hot hydrochloric acid under conditions known to cause removal of tritium from only the 3- and 5-positions resulted in no change in the $^{14}$C activity, but >99% loss of $^3$H. Thus conversion of phenylalanine into tyrosine in the intact *Pseudomonas* organism had occurred without "scrambling" of the radio-labels, and was accompanied by the NH shift of tritium. The results are summarised in Table 5.

Two independent sets of experiments were carried out, leading to $^{14}$C incorporations of ca. 6% and ca. 17%. The small differences between the two sets of results are not regarded as significant, and the retentions of tritium upon hydroxylation of $[4-^3\text{H}]$- and $[4-^3\text{H};3,5-^2\text{H}_2]$ phenylalanine are taken to be 95±1% and 74±1% respectively.

Two important conclusions can be drawn. The degree of migration of tritium is high (≥95%), and the degree of retention of tritium subsequent to migration depends markedly upon the nature of the isotopic species originally in the meta position. The latter conclusion militates strongly against the participation of an enzyme which stereospecifically removes a meta substituent, and suggests rather that the degree of retention following migration is dictated by a kinetic isotope effect.

The high retention of tritium with the para-tritiated, non-deuterated precursor provides reasonable grounds for making the approximation that migration of the isotope occurs to completion, so that the degree of retention of tritium with the dideuterated precursor can be regarded
as a direct measure of the value of $k_D/k_T$. A retention of $74 \pm 1\%$ corresponds to a $k_D/k_T$ of $2.8 \pm 0.1$ from which, using the Swain\textsuperscript{47} relationship, a value of $k_H/k_D = 10 \pm 1$ can be calculated.

Strictly speaking the degree of tritium retention which accompanies hydroxylation of the deuteriated precursor is a measure of $k_T^T/k_T^D$, where the symbol $k_T^T$ refers to the rate-constant for the breaking of the C--D bond when tritium occupies a position seminal to deuterium, and \textit{vice versa} for $k_T^D$. The quantity $(k_H/k_D)$ we wish to deduce can be conveniently regarded as $k_H^H/k_H^D$ for the purposes of this discussion. It is thus important to assess the magnitude and direction of the possible contributions made by secondary isotope effects to the observed tritium retention.

The situation with which we are concerned involves transfer of a proton from one base (the keto-form of a phenol) to another base (presumably water), with a transition state approximating to (XXI). If we consider a proton transfer process represented by $AH + B \rightarrow A + HB$ (ignoring charges) the most important factor which decides the primary isotope effect is the zero-point energy of the stretching vibration of the A--H bond. In the transition state the stretching vibration of A--H changes into motion along the reaction co-ordinate. This motion has no real frequency and therefore no zero-point energy. The simplest expression for the isotope effect is $k_H/k_D = \exp(\Delta E_0/kT)$, where $\Delta E_0$ is the difference in zero-point energies between A--H and A--D (the latter being the lower). Where the
proton is transferred from carbon, $\Delta E_0$ is ca. 1.15 Kcal. mole$^{-1}$, if the stretching vibration alone is considered, and a value of $k_H/k_D = 6.9$ is predicted at 25$^\circ$. This simple treatment predicts that $k_H/k_D$ should be independent of the nature of $B$, but experimental evidence indicates that the isotope effect does vary according to the strength of the base which is abstracting the proton.

Secondary isotope effects arise, in the case under consideration, because the carbon atom involved in the proton transfer undergoes rehybridisation at the transition-state. The most important factor which decides the secondary isotope effect is the activation energy involved in going from the ground-state to the transition-state, i.e. the increase in zero-point energy on going from the ground-state to the transition-state. This is decided principally by the zero-point energy of the out-of-plane $C-H$ bending vibration. This vibration at an $sp^3$-hybridised carbon atom has a higher frequency, and hence a higher zero-point energy, than that with $sp^2$-hybridisation. Typical values for the difference between the frequencies of the out-of-plane bending vibrations for $sp^3$- and $sp^2$-hybridisation are, for the $C-H$ bond, 600 cm$^{-1}$, and for the $C-D$ bond, 440 cm$^{-1}$. In other words decrease in $p$-character involves a release of energy (in this context), but less energy is released with $C-D$ than with $C-H$. Generally, for a carbon atom undergoing rehybridisation with decrease of $p$-character in its $C-H$ bonds at the transition-state, the rate of the step in the reaction sequence involving rehybridisation will be lowered by substitution of deuterium for protium.
The direction of the influence which secondary isotope effects would have on our value for $k_H/k_D$ can be deduced as follows. If secondary isotope effects were unity our measured value of $k_D^T/k_T^D$, derived from the loss of tritium from (XXII), would be equal to $k_H^T/k_T^H$, an expression for the true primary isotope effect. Taking as an example the rate of loss of protium from the situations (XXIII) and (XXIV), the C—H bond in (XXIV) will break at a slower rate than that in (XXIII), because rehybridisation with decrease in p-character will be retarded by the presence of the heavy isotope. Comparing situation (XXII) with situations (XXIV) and (XXV), $k_D^T$ will have a smaller value than $k_D^H$, and $k_T^D$ will have a smaller value than $k_T^H$. However, the percentage decrease in $k_D$ when T is substituted for H at the geminal position will be greater than the percentage decrease in $k_T$ when D is substituted for H. Hence $k_D^T/k_T^D$ will be smaller than $k_D^H/k_T^H$, so that secondary isotope effects would tend to make our calculated value for $k_H/k_D$ lower than the true value.

Secondary isotope effects are usually quite small relative to the primary effect. Kresge and Chiang found that for loss of hydrogen ion from the phenonium ion (XXVI) the apparent isotope effect $k_D^H/k_D^H$ was equal to $8.1 \pm 0.3$. This value was the quotient of the primary and secondary effects. A value of $1.11 \pm 0.03$ was deduced for the secondary effect $k_D^H/k_D^D$, so that the true primary isotope effect, $k_H/k_D$, would be given by

$$(k_H^D/k_D^H)(k_H^T/k_D^T) = (8.1 \pm 0.3)(1.11 \pm 0.03) = 9.0 \pm 0.4.$$
Hence the likely influence of secondary isotope effects would be to make our estimate for $k_H/k_D$ slightly low.

A chemical analogy for the enolisation of the cationic species (XVIII) is available from work by Russell$^{57}$. A value of $k_H/k_D = 7.2$ was observed in the rate of aromatisation of (XXVII). This is considerably lower than our value for the isotope effect in the case of phenylalanine, but the intermediate involved in the enolisation to give tyrosine may be of the type (XIX). A chemical model for this process exists in the form of the dimerisation of the radical (XXVIII) in neutral benzene, which has been studied by Mahoney and Weiner$^{58}$. Under these conditions the rate-determining step is believed to be the enolisation of a keto-form of the C-C coupled, phenolic product. Comparison of the rates of dimerisation of (XXVIII) and its 2,4,6-2H$_3$-derivative allowed a value of $10^{\pm 2}$ to be calculated for $k_H/k_D$. This inclines us to favour (XIX) rather than (XVIII) as the probable intermediate involved in the aromatisation to give tyrosine. Hence it is possible that the entire sequence of hydroxylation of phenylalanine under physiological conditions, beginning with the formation of the arene oxide, involves intermediates with no overall net charge.

The studies of Kasperek and Bruice$^{41}$ lend partial support to this proposition.

The strength of the base involved in the loss of the hydrogen species has been shown to affect the value of the kinetic isotope effect for hydrogen transfer. Bell and Crooks$^{59}$ reported that the isotope effect involved in the rate of ionisation of ethyl $\alpha$-methylacetate increased
with increase in the strength of the base. The overall reaction was bromination, which is zero-order with respect to bromine, the rate being dependent upon the ease of transfer of a proton to the base. Studies of hydrogen exchange in azulenes lend support to the hypothesis that the highest isotope effects are observed when the proton is transferred between bases of similar strengths. In the case of phenylalanine hydroxylation the base which abstracts the proton is presumably water (unless an enzyme-bound base is involved, which is not considered likely) and a transition state of the form (XXI) is envisaged.

We suggest that the hydroxylation of phenylalanine by Pseudomonas proceeds via the following steps: (i) enzymic generation of the 3,4-oxide; (ii) spontaneous opening of the oxirane ring and migration of para-hydrogen to produce the keto-form of tyrosine; and (iii) enolisation, accompanied by a high kinetic hydrogen isotope effect. There is no evidence for enzymic involvement beyond the first step.
Section B. Incorporation of Phenylalanine and Cinnamic Acid into Alkaloids of "Texas" Daffodile

The Amaryllidaceae alkaloids norpluviine\textsuperscript{60} and haemanthamine\textsuperscript{61} are known to arise biogenetically\textsuperscript{62-65} from phenylalanine and tyrosine via the common intermediate Q-methylnorbelladine. The aromatic ring of both alkaloids is derived from phenylalanine, and undergoes two hydroxylations, the first of which involves a cinnamate substrate and offers possibilities for study of the NIH shift. Following similar lines to those of the \textit{Pseudomonas} experiments we have studied the degree of retention of tritium accompanying the incorporation of \([4-3\text{H}]\) and \([4-3\text{H};3,5-\text{H}_2]\)-labelled specimens of cinnamic acid and phenylalanine into both norpluviine and haemanthamine. The incorporation of the corresponding meta-tritiated precursors has also been studied.

Preparation of the two para-tritiated phenylalanines has been described in Section A. DL-\([3-3\text{H}]\)Phenylalanine was a gift from Dr. N. Johns. Specimens of the ring-labelled cinnamic acids were prepared by treatment of the correspondingly-labelled phenylalanines with base and dimethyl sulphate. The position of tritium in DL-\([3-3\text{H}]\)phenylalanine was checked by degradation as described for the para-tritiated precursors (Section A), with an additional step involving bromination on \([3-3\text{H}]\)aniline to the 2,4,6-tribromo-\([3-3\text{H}]\)-derivative (Table 6).

The tritiated phenylalanines were each mixed with DL-phenylalanine uniformly-labelled with \(^{14}\text{C}\), to provide samples of known \(3\text{H}/^{14}\text{C}\) ratio. Similarly the tritiated cinnamic acids
TABLE 6

Degradation of cinnamic acid derived from DL-[3-³H]phenylalanine

<table>
<thead>
<tr>
<th>Molar activities relative to cinnamic acid = 1.00</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzoic Acid</td>
</tr>
<tr>
<td>1·01</td>
</tr>
</tbody>
</table>

were each mixed with $\left[\text{B-}^{14}\text{C}\right]$cinnamic acid (prepared by condensation of malonic acid with $\left[\text{carbonyl-}^{14}\text{C}\right]$benzaldehyde). The precursors were administered, in parallel, to flowering "Texas" daffodils. After 8 days' further growth the plants were harvested and macerated with ethanolic tartaric acid. The extracts were acidified, and the chloroform-soluble components were removed. After basification with sodium hydrogen carbonate the aqueous solution was extracted with chloroform, which on evaporation to a small volume yielded a precipitate of crude norpluviine, which was purified by dissolution in dilute aqueous sodium hydroxide, filtration of the solution, reprecipitation of the alkaloid with carbon dioxide, and recrystallisation from methanol. Haemanthamine was isolated from the chloroform-soluble alkaloid fraction by chromatography on alumina and recrystallisation from ethyl acetate.

The norpluviine was recrystallised to constant specific activity and $\text{H/}^{14}\text{C}$ ratio, then converted into the diacetyl derivative for further radioactive counting. The haemanth-
amine was recrystallised to constant specific radioactivity and $3\mathrm{H}/^{14}\mathrm{C}$ ratio, then converted into the picrate, which was crystallised and converted back into the free alkaloid (Table 7). In the cases where phenylalanine was administered the values of $3\mathrm{H}/^{14}\mathrm{C}$ quoted for the precursors refer to specimens of benzoic acid derived from the labelled phenylalanines by permanganate oxidation. This conveniently gave a derivative containing the $C_6-C_1$ unit which is incorporated into each of the alkaloids.

The utilisation of *Amaryllidaceae* alkaloid biosynthesis as a means of studying hydroxylation-induced migration of tritium is made possible by a number of important characteristics of the biosynthetic process. Barton and Cohen suggested$^{6,7}$ in 1957 that the variety of ring systems present amongst the *Amaryllidaceae* alkaloids could all arise biologically by oxidative cyclisation of phenolic precursors of the type represented by norbelladine (XXIX). Confirmation of this prediction arose as a result of elegant experimental work in the early 1960's. Battersby and his collaborators studied$^{6,4,6,8}$ the incorporation of $[^{14}\mathrm{C}]\alpha\text{-tyrosine}$ (XXX) into lycorine (XXXI) and haemanthamine (XXXII). Degradation of the alkaloids showed that the radioactive label in each case was restricted to the position marked with an asterisk. This indicated that the $C_6-C_2$ unit of each alkaloid was derived from tyrosine. $[^{14}\mathrm{C}]\text{Norbelladine}$ (XXXIII) was also incorporated$^{6,4,6,9}$ into lycorine and haemanthamine giving identical labelling patterns to those observed with $[^{14}\mathrm{C}]\alpha\text{-tyrosine}$. This gave support to the view that
<table>
<thead>
<tr>
<th>Precursor</th>
<th>$\mu$Ci $^{14}$C fed</th>
<th>Incorporation of $^{14}$C (%)</th>
<th>$^{3}$H/$^{14}$C ratio</th>
<th>Norpluviine</th>
<th>Haemanthamine</th>
<th>Overall mean $^{3}$H/$^{14}$C</th>
<th>Overall mean retention of $^{3}$H (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[3^{3}$H; $\beta^{14}$C] - cinnamic acid</td>
<td>7.90</td>
<td>11.87</td>
<td>0.57</td>
<td>5.66</td>
<td>5.70</td>
<td>5.68</td>
<td>5.71 47.9</td>
</tr>
<tr>
<td>$[4^{3}$H; $\beta^{14}$C] - cinnamic acid</td>
<td>6.70</td>
<td>8.17</td>
<td>0.089</td>
<td>3.48</td>
<td>3.48</td>
<td>3.48</td>
<td>3.54 43.0</td>
</tr>
<tr>
<td>$[\alpha^{3}$H; $\beta^{14}$C] - cinnamic acid</td>
<td>6.73</td>
<td>9.27</td>
<td>0.088</td>
<td>2.98</td>
<td>3.00</td>
<td>2.99</td>
<td>2.95 31.8</td>
</tr>
<tr>
<td>DL-[3$^{3}$H; $U^{14}$C] - phenylalanine</td>
<td>7.43</td>
<td>13.62</td>
<td>0.19</td>
<td>6.64</td>
<td>6.66</td>
<td>6.65</td>
<td>6.67 48.9</td>
</tr>
<tr>
<td>DL-[4$^{3}$H; $U^{14}$C] - phenylalanine</td>
<td>7.88</td>
<td>7.54</td>
<td>0.11</td>
<td>3.28</td>
<td>3.24</td>
<td>3.26</td>
<td>3.21 42.8</td>
</tr>
<tr>
<td>DL-[4$^{3}$H; $3,5^{2}$H$_2$; $U^{14}$C] - phenylalanine</td>
<td>8.57</td>
<td>7.47</td>
<td>0.15</td>
<td>2.57</td>
<td>2.39</td>
<td>2.48</td>
<td>2.36 32.4</td>
</tr>
</tbody>
</table>
phenolic coupling was an important step in the biosynthetic pathway. Barton et al. made more intensive investigations of the intermediacy of diphenols of the norbelladine type by studying the incorporation of the $^{14}$C$_3$-labelled precursor (XXXIV) into galanthamine (XXXV). The labelling pattern in the alkaloid showed complete absence of "scrambling" of the $^{14}$C, and that neither of the bonds to the nitrogen atom in the precursor were broken before incorporation. Studies by Jeffs of the incorporation of $[\beta^{14}$C$_2$]tyrosine (XXXVI) into haemanthamine provided confirmation of the origin of the C$_6$—C$_2$ unit, and showed that tyrosine did not provide the C$_6$—C$_1$ unit.

Seeking a precursor for the C$_6$—C$_1$ unit, Wildman et al. found that $[\alpha$-C$_2$]phenylalanine (XXXVII) was incorporated into lycorine and belladine (XXXVIII) in a specific manner, the label being located only at the benzylic carbon atom. Thus phenylalanine could serve as a precursor for the C$_6$—C$_1$ unit, but not for the C$_6$—C$_2$ unit.

Suhadolnik and his co-workers independently confirmed this finding for lycorine and haemanthamine, and proceeded to investigate some of the enzymes relevant to the biosynthetic pathway. The presence of phenylalanine ammonia-lyase, which catalyses the deamination of phenylalanine to trans-cinnamic acid, was demonstrated in Amaryllidaceae plants, and it was shown that $[\beta$-C$_2$]cinnamic acid, 4-hydroxy-$[\beta$-C$_2$]cinnamic acid, and protocatechuic aldehyde (XXXIX) were incorporated into the
(XXXVII)

(XXXVIII)

(XXXIX)
Thus a fairly complete picture of the biosynthesis of the Amaryllidaceae alkaloids has been constructed (Scheme 13). Protocatechuic aldehyde, derived from phenylalanine, condenses with tyramine (XL) to give the Schiff's base (XLI), which is reduced and methylated to yield o-methylnorbelladine (XLII). Para-ortho coupling of the latter leads to norpluviine (XLIII), while para-para coupling leads ultimately to haemanthamine.

Specimens of phenylalanine and cinnamic acid, labelled with tritium at the 4-position, would thus furnish the aromatic ring of both norpluviine and haemanthamine. The ring would undergo two hydroxylations, the first at the step cinnamic acid $\rightarrow$ p-coumaric acid (XLIV), presumably accompanied by an NIH shift of tritium, and the second at the step p-coumaric acid $\rightarrow$ caffeic acid (XLV). By analogy to the conversion of tyrosine to 3,4-dihydroxy-phenylalanine the latter step would not be accompanied by an NIH shift, but would result in complete elimination of the hydrogen isotope from the position of hydroxylation. Hence any tritium remaining in the resulting alkaloids should be located at the 11-position of norpluviine, or the (equivalent) 10-position of haemanthamine. This expectation was tested by performing an electrophilic substitution at the 8-position of norpluviine. The alkaloid is known to form a monobromo-derivative which was characterised in the form of the diacetate. We confirmed that the bromine had substituted at the 8-position as follows. Norpluviine was treated with a solution of
sodium deuterioxide in deuterium oxide at 100° for 7 days, conditions which would cause hydrogen exchange ortho- to the phenolic hydroxyl group. A portion of the product was converted into the diacetyl derivative, and the n.m.r. spectrum compared with that of diacetylnorpluviine, which shows two aromatic proton resonances, at $\tau 3.17$ and $\tau 3.27$. The spectrum of the deuteriated compound lacked the upfield resonance. Bromination of the deuteriated norpluviine, and formation of the diacetyl derivative did not cause the remaining aromatic resonance to disappear, thus confirming that bromine had entered the 8-position (Scheme 14).

A sample of norpluviine derived biogenetically from a para-tritiated cinnamic acid precursor was then converted to the 8-bromodiacetyl derivative without loss of tritium activity. Hence the retention of tritium which accompanies the incorporation of meta- and para-tritiated phenylalanine and cinnamic acid represents half the retention associated with the first hydroxylation of the ring.

The data of Table 7 reveal a striking similarity between the tritium retentions for the phenylalanine series and those for the cinnamic acid series, and add weight to the large body of evidence that the aromatic rings of both precursors are hydroxylated in the form of a common substrate. In view of the close similarity between the two sets of results, the mean values of tritium retention which will be used for the purposes of this discussion are those listed in Table 8. It follows that the retention of tritium at the first hydroxylation is, for a para-tritiated precursor, 35.8 $\pm 0.6\%$ when the tritium is flanked by protium;
Scheme 14

\[
\begin{align*}
\text{MeO} & \quad \text{HO} \\
\text{Ac} & \quad \text{D} \\
\text{pyridine} & \quad \tau 3.17 (H_a), 3.27 (H_b)
\end{align*}
\]

\[
\begin{align*}
\text{MeO} & \quad \text{HO} \\
\text{D} & \quad \text{Br}
\end{align*}
\]

\[
\begin{align*}
\text{MeO} & \quad \text{HO} \\
\text{Ac} & \quad \text{D} \\
\text{pyridine} & \quad \tau 3.17 (H_a)
\end{align*}
\]

\[
\begin{align*}
\text{MeO} & \quad \text{HO} \\
\text{Br} & \quad \text{Ac}
\end{align*}
\]

\[
\begin{align*}
\text{MeO} & \quad \text{HO} \\
\text{Ac} & \quad \text{D} \\
\text{pyridine} & \quad \tau 3.13 (H_a)
\end{align*}
\]
TABLE 8
Retention of tritium on incorporation of phenylalanine and cinnamic acid into norpluviine and haemanthamine

<table>
<thead>
<tr>
<th>Isotopic hydrogen labelling pattern in precursor</th>
<th>Mean retention of tritium (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^3\text{H}$</td>
<td>$48.4 \pm 0.3$</td>
</tr>
<tr>
<td>$^4\text{H}$</td>
<td>$42.9 \pm 0.3$</td>
</tr>
<tr>
<td>$^4\text{H}; {^3,^5\text{H}_2}$</td>
<td>$32.1 \pm 0.3$</td>
</tr>
</tbody>
</table>

and $64.2 \pm 0.6\%$ when the tritium is flanked by deuterium. For a meta-tritiated precursor the tritium retention at the first hydroxylation is $96.8 \pm 0.6\%$. In the latter case, since both meta positions are equivalent, and tritium is present at only one of them, the percentage loss of the tritium involved in the enolisation process is double the overall loss at the first hydroxylation; i.e. the corrected retention is $93.6 \pm 1.2\%$.

The relevance of these data to the question of the possible involvement of an arene oxide isomerase can be appreciated if one considers that stereospecific removal of an isotope ortho to the position of hydroxylation would be expected to produce the following overall tritium retentions in the biosynthesis of each alkaloid: for a para-tritiated precursor, irrespective of the nature of the isotope occupying the meta position, ca. $50\%$; for a meta-tritiated precursor, ca. $25\%$. The present findings are clearly at variance with those of previous experiments.  

53.
and do not provide any evidence for the existence of an arene oxide isomerase, but indicate rather that the loss of tritium is strongly influenced by a kinetic isotope effect. Feeding of a \([3-^3H]\)-labelled precursor provides the most sensitive test for a stereospecific elimination process. Our results show that a greater proportion of the tritium is retained upon hydroxylation of a meta-tritiated precursor than when a para-tritiated precursor is employed; i.e., there is a reversal of the order expected if an isomerase of the type postulated were involved.

The observation that tritium retention is greater when the isotope is initially at C-3 than when it has to migrate to that position suggests that a portion of the loss of the para substituent occurs without migration; that is, by a "direct loss" mechanism (cf. path b in Scheme 2). Comparison of these results with those obtained with Pseudomonas shows that the retention of tritium on hydroxylation of para-tritiated cinnamate is ca. 9% lower than on hydroxylation of para-tritiated phenylalanine. A similar drop in tritium retention is evident when the results with the \([4-^3H; 3,5-^2H_2]\)-labelled substrates are compared (74% retention in the case of phenylalanine hydroxylation in Pseudomonas, 64% in the case of cinnamate hydroxylation in "Texas" daffodils).

The involvement of a "direct loss" pathway precludes the use of the result obtained from the dideuteriated precursors to calculate \(k_D/k_T\) directly. However, the ratio of the mean tritium retention with the \([4-^3H]\)-precursors to that with the \([4-^3H; 3,5-^2H_2]\)-precursors can be used to
estimate the isotope effect as follows. Considering the hydroxylation of a \([\text{L-}^3\text{H}]\)-precursor (Scheme 15) we will let \(x\) be the fraction of tritium undergoing migration, so that \((1-x)\) is the fraction eliminated without migration. Further, let \(y\) be the fraction of the migrated tritium which is retained upon aromatisation of the keto form of the phenol. Then the fraction of the original tritium which is retained overall will be \(xy\), which according to our data has the value 0.858. Similarly, on the assumption that the degree of migration is not influenced by a secondary isotope effect, the fraction of tritium which migrates on hydroxylation of the \([\text{L-}^3\text{H}; 3,5-^2\text{H}_2]\)-precursor will also be \(x\), while the fraction of tritium retained on isomerisation of the cyclohexadienone to the phenol we shall denote by \(z\). In this case the fraction of the original tritium retained overall will be \(xz\), numerically equal to 0.642.

The fraction of tritium, \(y\), retained in competition with protium at the enolisation step will be determined by the ratio of the rate of protium loss, \(k_H\), to the overall rate of aromatisation, \(k_H + k_T\). That is

\[
y = \frac{k_H}{k_H + k_T},
\]

which we can rewrite as \(\frac{1}{y} = 1 + \frac{k_T}{k_H} \cdots \text{(i)}\)

Similarly the fraction of tritium retained in competition with deuterium at the enolisation step can be expressed as

\[
z = \frac{k_D}{k_D + k_T},
\]

which can be rewritten as \(\frac{1}{z} = 1 + \frac{k_T}{k_D} \cdots \text{(ii)}\)

From the Swain\(^{47}\) relationship, \(\frac{k_D}{k_T} = (k_H/k_T)^{0.306}\).

Hence equation (ii) can be rewritten
SCHEME 15

Fractional migration and retention of tritium

\[
\text{Scheme diagram showing migration and retention of tritium.}
\]
If we now denote \( \frac{k_H}{k_T} \) by the symbol \( k \), equation (i) becomes

\[
\frac{1}{z} = 1 + \frac{1}{(\frac{k_H}{k_T})^{0.306}}
\]

and equation (ii) becomes

\[
\frac{1}{z} = 1 + \frac{1}{k^{0.306}}.
\]

Hence

\[
\frac{1}{z} = 1 + \frac{1}{k^{0.306}}
\]

Denoting the fraction \( \frac{y}{z} \) by the symbol \( r \), we have

\[
r + \frac{r}{k} = 1 + \frac{1}{k^{0.306}}
\]

or,

\[
kr + r = k + k^{0.6935} .... (iii).
\]

We thus have a relationship between two important quantities: \( r \), the ratio of tritium retention with a \([4-\text{H}]\)-precursor to tritium retention with a \([4-\text{H}; 3,5-\text{D}]\)-precursor, and \( k \), the tritium isotope effect, \( k_H/k_T \). Note that secondary isotope effects are here assumed to be unity, a limitation which is imposed by use of the Swain relationship. According to the data from the present work, \( r = 0.858/0.642 = 1.336 \), and solution of equation (iii) for \( k \) should provide the corresponding value of \( k_H/k_T \). However, the equation cannot be solved directly, and to appreciate the nature of the relationship between \( k \) and \( r \) it is necessary to compute a number of values and interpolate graphically. The computations, kindly carried out by Dr. B.C. Webster (Glasgow), are detailed in Table 8, and are displayed graphically in Fig. 3. \( r \) has a maximum value of 1.359, when \( k = 8.564 \). For all other values of \( r \) there are two corresponding values for \( k \).
TABLE 8

<table>
<thead>
<tr>
<th>( r )</th>
<th>( k )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>1.1</td>
<td>1.36</td>
</tr>
<tr>
<td>1.16</td>
<td>1.67</td>
</tr>
<tr>
<td>1.23</td>
<td>2.23, 102.4</td>
</tr>
<tr>
<td>1.27</td>
<td>2.73, 54.76</td>
</tr>
<tr>
<td>1.30</td>
<td>3.31, 34.56</td>
</tr>
<tr>
<td>1.34</td>
<td>4.85, 17.28</td>
</tr>
<tr>
<td>1.35</td>
<td>5.72, 13.60</td>
</tr>
<tr>
<td>Maximum</td>
<td>1.359, 8.564</td>
</tr>
</tbody>
</table>

Applying our experimental value for \( r \) (1.336) we find that \( k \) can have the values 4.60 or 18.74. For our purposes the relevant value for \( k \) can be selected by considering the values of \( y \) and \( z \) which correspond to each of the two possible values of \( k \). From equation (i), if \( k = 4.60 \), \( y = 0.821 \); if \( k = 18.74 \), \( y = 0.949 \). Since we have defined \( y \) as the fraction of tritium retained in competition with protium at the enolisation step, and the experimental value for \( x \) is 0.858, we must select 18.74 as the correct value for \( k \), since \( y \) cannot be \(< 0.858 \). Similarly, from equation (ii), values of 4.60 and 18.74 for \( k \) lead to values of 0.615 and 0.711 respectively for \( z \), and again, we must select the second value of \( k \), since \( z \) cannot be \(< 0.642 \), our experimental value for \( xz \). We
FIG. 3

\[ r = k^{0.6935} - k(r-1) \]
have thus arrived at a value of 18.74 for the tritium isotope effect $k_{H}/k_{T}$. Random error limits of ±0.006 in our values for $y$ and $z$ lead to limits of ±0.015 in the value of $r$, and hence $k$ would lie between 13.6 and 23.4. We therefore propose a value of 19 ± 5 for $k_{H}/k_{T}$, which, by application of the Swain relationship, corresponds to a $k_{H}/k_{D}$ of 7.7 ± 1.4. This is lower than the value for $k_{H}/k_{D}$ (10 ± 1) obtained from the Pseudomonas experiments, but the difference may not be significant, as the ranges contained within the error limits overlap at a $k_{H}/k_{D}$ of ca. 9. However, there is no reason to expect that the isotope effect associated with the enolisation of an intermediate based on cinnamate will be identical to that of the phenylalanine analogue. It is interesting to note that when we apply the above treatment to the results from Pseudomonas, $r$ has a value of 1.285 ± 0.022, which corresponds to a $k_{H}/k_{T}$ of 44, with a lower error limit of 30, and a $k_{H}/k_{D}$ of 13.8, with a lower error limit of 10.6. The latter value lies within our limits for $k_{H}/k_{D}$ (10 ± 1) derived from tritium retention with the deuteriated precursor alone. The difference in the mean values for $k_{H}/k_{D}$ obtained by the two different methods of calculation may reflect the magnitude of experimental error, but probably also serves to indicate that secondary isotope effects are not without significance, either at the enolisation step as discussed earlier, or at the migration step. The assumption we made in connection with the Pseudomonas data, that migration of the isotope is complete, may be the source of a small error in the value of $r$. A
further small error might arise if the fraction \(x\) of tritium which migrates is different when deuterium rather than protium occupies the adjacent position on the ring. The overall conclusion remains the same, namely that the aromatisation process following the NIH shift in Pseudomonas shows a high isotope effect, \(k_H/k_D\) being ca. 10.

An interesting comparison can be made with a recently published report\(^7\) of work with \([4-\text{H}_2]_4\) and \([4-\text{H}; 3,5-\text{H}_2]_4\)-labelled specimens of cinnamic acid, anisole, and acetanilide. Retention of tritium during hydroxylation of the cinnamic acids by chick pea microsomes produced a value for \(r\) of 1.33, showing good agreement with our observations for cinnamate hydroxylation \textit{in vivo}. The corresponding value of \(r\) for hydroxylation of the labelled anisoles, microsomally or \textit{in vivo} (rodents) was 1.26. However, hydroxylation of \([4-\text{H}_2]_4\) and \([4-\text{H}; 3,5-\text{H}_2]_4\)-acetanilide in the same rodent systems led to an \(r\) value of 1.54 - 1.55. This is surprising in view of the maximum value of 1.359 which arises from equation (iii), and again raises the question of possible secondary isotope effects. The authors do not make any comment on the possible margins of error associated with their data. The high \(r\) value was obtained using a substrate with a strongly electron-donating side-chain, leading to a high loss of tritium without migration. Consequently the overall tritium retentions were relatively low: 40% with \([4-\text{H}]\text{acetanilide, and 26% with [4-\text{H}; 3,5-\text{H}_2]}\text{acetanilide, in vivo. Furthermore, the hydroxylation of acetanilide appears to be unique in a number of ways. The} \)
retention of deuterium during hydroxylation of $[4\cdot 2^2H]$-acetanilide has been shown\textsuperscript{12} to be dependent upon the source of the microsomes (e.g. rats, mice, or rabbits), and upon the type of pre-treatment (e.g. phenobarbital or benzpyrene) given to the animal. Retention of isotope in vitro was found\textsuperscript{74} to be markedly higher than that in vivo. The existence of two "acetanilide hydroxylases" has been postulated\textsuperscript{12,74} as an explanation of these anomalies.

We have already noted that a comparison of the retentions of tritium when $[4\cdot 3^2H]$- and $[3\cdot 3^2H]$-cinnamic acid are hydroxylated indicates that a "direct loss" pathway is involved; i.e. rearrangement of an arenal oxide to the corresponding phenol occurs by elimination of the para substituent without an NIH shift. This "non-migration" loss accounts for some 9\% of the total loss of tritium, and suggests that the side-chain of cinnamate can interact mesomerically with the ring, thus delocalising the positive charge generated by opening of the epoxide. This would facilitate re-aromatisation via direct elimination of the para substituent. This type of behaviour has been illustrated in the case of acetanilide (Scheme 3). The non-migration loss of tritium which accompanies the hydroxylation of $[4\cdot 3^2H]$-acetanilide is of the order of 50\% at pH 8. Clearly this is a much higher direct loss than that which occurs with $[4\cdot 3^2H]$cinnamic acid, but it probably reflects the weaker electron-donating character of the side-chain of the latter. The negative charge on the carboxylate group will encourage the donation of electrons to the ring, but the
SCHEME 16

\[
\begin{align*}
\text{Scheme diagram with molecular structures and reactions.}
\end{align*}
\]
\[ \text{polarisation will oppose donation to some extent (Scheme 16).} \]

If the difference between the values of $k_R/k_D$ for the keto-enol rearrangement when phenylalanine is hydroxylated \((10 \pm 1)\) and that when cinnamate is hydroxylated \((7.7 \pm 1.4)\) is significant, one might speculate that the difference arises as a result of the different electronic properties of the two side-chains. The isotope effect would then be lowered by an electron-releasing substituent \((i.e., \text{by an increase in the basic strength of the ring})\).

However, the available data are far too sparse to support such an argument. Furthermore, when compounds with strongly electron-donating substituents are hydroxylated the direct loss mechanism predominates, making isotopic retention measurements subject to greater errors.
EXPERIMENTAL

Infra-red spectra were determined for potassium bromide discs using a Perkin Elmer 257 grating spectrophotometer. $^1$H nuclear magnetic resonance spectra were determined at 60 MHz, using tetramethylsilane as internal standard, with a Perkin Elmer R.10 spectrometer. Mass spectra were determined using an A.E.I. M.S.12 spectrometer. Melting points were determined on a Kofler hot-stage apparatus, and are uncorrected.

Analytical chromatography of amino acids was carried out on Whatman No. 1 paper, using a solvent system of $n$-butanol (60 parts), acetic acid (15 parts), and water (25 parts). The amino acids were rendered visible by spraying with ninhydrin solution followed by heating at 110°C. In the case of a labelled sample, radioactivity was located by scanning the chromatogram, prior to ninhydrin treatment, on a Panax model RTLS - 1A scanner equipped with a windowless Geiger-Muller tube supplied with argon containing 2% of propane.

Centrifugation of bacterial cell suspensions was carried out in an MSE Multex centrifuge operating at 4000 r.p.m. for 45 mins.

Solvents were dried by standard methods where appropriate. All compounds described are colourless solids unless otherwise stated. Solutions of products in organic solvents were dried over anhydrous sodium sulphate, and the solvent removed by evaporation in vacuo.
Counting of radioactive compounds

$^3$H and $^{14}$C activities were measured with a Beckman Instrument CFM-100 liquid scintillation spectrometer. With the exception of the amino acids, a sample (typically 0.5 mg.) of the compound was dissolved in dimethylformamide (1 ml.) in a counting vial, and toluene-based scintillation solution (10 ml., containing 0.38% 2,5-diphenyloxazole and 0.02% 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene was added. Phenylalanine and tyrosine were converted into their respective hydrochlorides by dissolving a sample of the amino acid in ethanolic hydrogen chloride in a counting vial and evaporating the solvent in vacuo, over potassium hydroxide, for a minimum of 2h. The residue was dissolved in dimethylformamide and toluene-based scintillation added as above. The sample was counted immediately after preparation.

In the case of a sample containing both $^3$H and $^{14}$C the activities of the two isotopes were determined by dividing the energy spectrum into two channels, so arranged that virtually all the $^3$H activity was confined to the lower channel (Channel 1). A major portion of the $^{14}$C activity was detected in the upper channel (Channel 2), with a smaller portion being detected in Channel 1. The individual activities of the isotopes were calculated as follows. Let $C_I$ be the number of counts per minute (corrected for background) in Channel 1, $C_{II}$ the number of counts per minute (corrected for background) in Channel 2, $I_I$ and $I_C$ the efficiencies of counting of $^3$H and $^{14}$C respectively in Channel 1, and $I_C$ the efficiency of counting of $^{14}$C in Channel 2; then
disintegrations per minute of $^{14}\text{C} = \frac{\text{C}_{\text{II}}}{\text{I}_{\text{C}}}$, and

\[ ^{3}\text{H} = \frac{\text{C}_{\text{I}} - \frac{\text{C}_{\text{II}}}{\text{I}_{\text{C}}}}{\text{I}_{\text{T}}} \]

Counting efficiencies were determined using specimens of hexadecane, separately labelled with $^{3}\text{H}$ and $^{14}\text{C}$, of known specific activity, obtained from the Radiochemical Centre, Amersham. For each substance to be counted, efficiencies were determined in the presence of the non-radioactive compound, in order that allowance could be made for quenching.

**DL-[4-$^{3}\text{H}$]Phenylalanine**

(a) [4-$^{3}\text{H}$]Toluene. An excess of lithium (0.25 g.) was added in small fragments to a stirred solution of 4-iodotoluene (3.27 g., purified by sublimation) in dry ether (40 ml.) under an atmosphere of nitrogen. After 3 h., tritiated water (0.5 ml., 3.6 mCi/mole$^{-1}$) was added through a serum cap. After a further 20 min. stirring the mixture was diluted with water, and the aqueous layer extracted with ether (3×10 ml.). The combined ethereal solutions were dried and concentrated by distillation at constant temperature through a short fractionating column. Carbon tetrachloride (20 ml.) was added to the concentrate and the last traces of ether were distilled off. The volume of the solution was made up to 20 ml. with carbon tetrachloride, and the concentration of [4-$^{3}\text{H}$]toluene (90% yield) was measured by h.m.r. by comparison with a standard solution.

(b) [4-$^{3}\text{H}$]Benzyl bromide. Bromine (0.8 ml.) in carbon tetrachloride (20 ml.) was added to the solution of
\[ [4-3^\text{H}] \text{toluene from (a) at such a rate that bromine did not persist in the mixture while the latter was irradiated with tungsten lamps (2x150 watts). The resulting solution was washed with water (3x10 ml.), dried, and evaporated to ca. 3 ml. Distillation at ambient pressure gave } [4-3^\text{H}] \text{benzyl bromide (1.09 g.).} \]

(a) 3-Phenylhydantoin. Glycine (9.45 g.), sodium hydroxide (5 g.), and water (40 ml.) were stirred together at ambient temperature until a clear solution was obtained. Phenylisocyanate (9.8 ml.) was added over 12 min., during which time the temperature rose to 55°. After stirring for a further 15 min. the mixture was heated at 70° for 15 min. Concentrated hydrochloric acid (15 ml.) was added, and the resulting suspension was stirred and cooled to 5°. The white crystals thus obtained were heated with concentrated hydrochloric acid (50 ml.) and the resulting clear solution was heated under reflux for 1 h. Removal of the solvent in vacuo and recrystallisation of the residue from ethanol gave 3-phenylhydantoin (11.8 g., 75%), m.p. 156-70° (lit. 159-60°, 75 154-5°76), \( \nu_{\text{max}} \) 3230 br, 1777m, 1725s cm\(^{-1}\), \( \gamma (\text{CDCl}_3) \) 2.48-2.70 (5H, arom.), 2.91 br (NH, disappears with \( \text{D}_2\text{O} \)), 6.02s (upfield shoulder, 2H).

(d) Magnesium methyl carbonate. Magnesium turnings (7.2 g.) were added cautiously to dry methanol (150 ml.) and the resulting solution was heated under reflux for 1.5 h. Most of the methanol was removed in vacuo at 45°. Dry dimethylformamide was added to the residue to give a total
volume of 150 ml., most of the remaining methanol was removed by fractional distillation, and dry carbon dioxide was passed into the suspension. When all the solid had dissolved the last traces of methanol were removed by distillation through a short fractionating column. The resulting solution of magnesium methyl carbonate (ca. 2M with respect to magnesium) was cooled under an atmosphere of carbon dioxide to ensure saturation.

(e) 5-\(\left[4-^3\text{H}\right]\)Benzyl-3-phenylhydantoin. A solution of 3-phenylhydantoin (1.1 g.) in magnesium methyl carbonate solution (13 ml.) was stirred under anhydrous conditions at 80° for 2 h. \(\left[4-^3\text{H}\right]\)Benzyl bromide (1.09 g.) was added and the mixture was stirred for 3.5 h. at 90-100°. Addition of the solution to ice/hydrochloric acid and recrystallisation of the resulting precipitate from acetone gave 5-\(\left[4-^3\text{H}\right]\)benzyl-3-phenylhydantoin (0.3 g.), m.p. 171-2° (lit. 170-2°), \(\nu_{\text{max}}\) 3260 br, 1780 m, 1725 s cm\(^{-1}\), \(\gamma\) (CDCl\(_3\)) 3.54-3.00 (1OH, arom.), 3.13 br (NH, disappears with D\(_2\)O), 5.72t (1H, J 6Hz), 6.99 (2H, J 6Hz).

(f) DL-\(\left[4-^3\text{H}\right]\)Phenylalanine. 5-\(\left[4-^3\text{H}\right]\)Benzyl-3-phenylhydantoin (140 mg.) was dissolved in a mixture of dioxan (5 ml.) and saturated aqueous barium hydroxide (10 ml.) and heated in a sealed tube at ca. 170° for 1.3 h. The tube was allowed to cool, the contents were removed, diluted with water to 100 ml., and barium removed as the sulphate at pH 6. The solution was evaporated to dryness, the residue was washed with boiling chloroform (10 ml.), and dissolved in a small quantity
of hot water. Addition of ethanol (dropwise) and cooling of
the resulting solution overnight in the refrigerator gave
DL-[\(4-^3H\)] phenylalanine (46 mg., 1.47 \(\text{mC} \text{mmole}^{-1}\)), which was
recrystallised from water-ethanol.

DL-[\(4-^3H; 3,5-^2\text{H}_2\)] Phenylalanine

(a) 4-Amino [\(3,5-^2\text{H}_2\)] toluene hydrochloride. A solution
of 4-aminotoluene hydrochloride (3.5 g.) in deuterium oxide
(2.5 ml.) was heated under reflux under nitrogen for 48 h.
The solvent was removed in vacuo, a further portion of deuterium
oxide (2.5 ml.) was added to the residue, and the solution was
heated for a further 48 h. This procedure was repeated until
a total of five exchanges had been carried out. The final
residue was dissolved in ethanol and treated with activated
carbon. Separation of the carbon, addition of ether to the
solution, and recrystallisation of the resulting precipitate
from ethanol-ether gave 4-amino [\(3,5-^2\text{H}_2\)] toluene hydro-
chloride (2.23 g.) \(\gamma\) (free amine in CDCl\(_3\)) 3.04s (2H, arom.),
6.59s (2H, -NH\(_2\)) disappears with D\(_2\)O), 7.80s (3H, -CH\(_3\)).
4-Aminotoluene (before exchange), \(\gamma\) (CDCl\(_3\)) 3.09d (2H, 7.8Hz),
3.54d (2H, 7.8Hz).

(b) 4-Iodo [\(3,5-^2\text{H}_2\)] toluene. Sodium nitrite (1.14g.)
in water (2.5 ml.) was added slowly to a stirred solution of
4-amino [\(3,5-^2\text{H}_2\)] toluene (2.23 g.) in 3N-hydrochloric acid
(10 ml.) at -5\(^0\) until a slight excess of nitrite was present
(starch-iodide paper). A solution of potassium iodide (2.7 g.)
in water (2.7 ml.) was added slowly, the temperature being
maintained in the range -5\(^0\) to 0\(^0\). After complete addition
the resulting solution was warmed gradually to 80°, treated with a little sodium bisulphite, rendered alkaline with sodium hydroxide, and steam distilled. Sublimation of the distillate in vacuo gave 4-iodo \( [3,5-^2\text{H}_2] \) toluene (2.53 g., 56%, m.p. 36° (lit. 36-70°).

(c) \([4-^3\text{H}; 3,5-^2\text{H}_2]\) Phenylalanine. 4-Iodo \([3,5^2\text{H}_2]\) -toluene was converted, by procedures similar to those described above, to \([4-^3\text{H}; 3,5-^2\text{H}_2]\) phenylalanine. m/e 169 (0.1%), 168(0.6%), 167(M+, 2.7%), 166(0.8%), 165(0.1%).

C.f. phenylalanine m/e 167(0.033%), 166(0.3%), 165(M+, 1.9%), 163(0.08%).

**Ring-labelled cinnamic acids.** Specimens of \([3-^3\text{H}];\), \([4-^3\text{H}];\), and \([4-^3\text{H}; 3,5-^2\text{H}_2]\) -cinnamic acid were each prepared by treatment of the corresponding labelled phenylalanine with an excess of aqueous sodium hydroxide and dimethyl sulphate. In a typical conversion the labelled phenylalanine (30 mg.), dissolved in 4N-sodium hydroxide (1.5 ml.) was stirred in the presence of dimethyl sulphate (0.7 ml.) at 0° for 2 h. Sodium hydroxide (0.25 g.) was added, and the mixture was heated slowly to 100°, and maintained at this temperature for 2 h. After cooling the reaction mixture was diluted with water (10 ml.), acidified with hydrochloric acid, and extracted with chloroform (5x5 ml.). Evaporation of the organic phase, sublimation (130°, 0.1 Torr) of the residue, and recrystallisation from aqueous methanol gave cinnamic acid (15 mg., 56%), m.p. 133° (lit. 77 133°).
\[ \text{[B-14C]} \text{Cinnamic acid.} \quad \text{[Carbonyl-14C]} \text{Benzaldehyde} \]

(30 mg., 50 Ci, Radiochemical Centre, Amersham), malonic acid (66 mg.), pyridine (0.5 ml.), piperidine (0.05 ml.), and sodium sulphate (25 mg.) were heated together under anhydrous conditions at 100° for 2 h. under nitrogen. The resulting solution was cooled, diluted with water (10 ml.), acidified with hydrochloric acid, and extracted with chloroform (5x5 ml.)

After evaporation of the solvent the residue was sublimed and recrystallised from aqueous methanol to yield \( \text{[B-14C]} \) cinnamic acid (30 mg., 72%), m.p. 133° (lit. 133°).

**Racemisation of uniformly-labelled L-[\text{14C}]phenylalanine.**

Uniformly-labelled L-[\text{14C}]phenylalanine (11 mg., 50\( _{\text{u}} \)Ci, Radiochemical Centre, Amersham) dissolved in 10N-hydrochloric acid (1 ml.) was heated in a sealed tube at 180° for 18 h. (In a control experiment treatment of non-radioactive L-phenylalanine under similar conditions resulted in complete racemisation). After cooling to 20° the solvent was evaporated in vacuo, the residue was dissolved in water (10 ml.), and the pH of the solution was adjusted to 6 with aqueous sodium hydroxide. Portions of the solution (2 ml. per pot of ca. 4 bulbs) were fed to "Texas" daffodils.

**Degradation of radioactive precursors.** Specimens of \( [3-\text{3H}] \), \( [4-\text{3H}] \), and \( [4-\text{3H}; 3,5-\text{2H}_2] \)-cinnamic acid, derived from the corresponding phenylalanines, were each isotopically diluted with non-radioactive cinnamic acid, and converted to benzoic.
acid, dinitrobenzoic acid, acetanilide, and 4-bromoacetanilide, the molar activity of tritium being determined for each derivative. The identity of each compound was confirmed by comparison of the i.r. spectrum with that of an authentic specimen. The procedure is exemplified by the case of \([4\text{-}^3\text{H}]\)cinnamic acid as follows.

(a) \([4\text{-}^3\text{H}]\)Benzoic Acid. A solution of \([4\text{-}^3\text{H}]\)cinnamic acid (900 mg., 3.25x10^6 dpm 3H, mmole\(^{-1}\)) in 10% aqueous sodium hydroxide (60 ml.) was treated with 5% aqueous potassium permanganate (100 ml.) at 100° for 5 h. After cooling to 20°, treatment with a small quantity of sodium bisulphite, and acidification with hydrochloric acid, the mixture was extracted with chloroform (5x20 ml.). Evaporation of the organic phase followed by sublimation (110°, 0.1 torr) of the residue and recrystallisation from water gave \([4\text{-}^3\text{H}]\)benzoic acid (560 mg., 76%, 3.32x10^6 dpm 3H, mmole\(^{-1}\)), m.p. 122° (lit. 122°).

(b) 3,5-Dinitro-\([4\text{-}^3\text{H}]\)benzoic acid. \([4\text{-}^3\text{H}]\)Benzoic acid (150 mg., 3.32x10^6 dpm 3H, mmole\(^{-1}\)) was dissolved in concentrated sulphuric acid (1.2 ml.) in a flask fitted for reflux. Fuming nitric acid (0.6 ml.) was added slowly, and the resulting mixture was heated to 100° over a period of 20 min., maintained at this temperature for a further 20 min. then heated at 130-140° for 1.5 h. The resulting solution was cooled to 20°, poured into ice-water (30 g.), neutralised with sodium hydrogen carbonate, and re-acidified with dilute sulphuric acid. Extraction with chloroform (5x5 ml.), evaporation of the solvent, and recrystallisation of the residue from aqueous ethanol gave 3,5-dinitro-\([4\text{-}^3\text{H}]\)benzoic
acid (170 mg., 65%, 3.27x10^6 dpm \(^{3}\text{H}\) mmole\(^{-1}\)), m.p. 205-7^\circ (lit. 77 207^\circ).

(c) [\(4-^{3}\text{H}\)] Acetanilide. Sodium azide (160 mg.) was added, over a period of 40 min., to a rapidly stirred solution of [\(4-^{3}\text{H}\)] benzoic acid (250 mg., 3.32x10^6 dpm \(^{3}\text{H}\) mmole\(^{-1}\)) in a concentrated sulphuric acid (2 ml.) at 40^\circ. The mixture was heated cautiously to 80^\circ, and maintained at this temperature for 3 h. The mixture was cooled, poured on to ice (10 g.), basified with sodium hydroxide, and extracted with chloroform (5x5 ml.). A portion of the resulting solution, representing 78% of the total quantity of [\(4-^{3}\text{H}\)] aniline formed, was warmed gently in vacuo to remove most of the chloroform. Concentrated hydrochloric acid (0.12 ml.), water (5 ml.), and acetic anhydride (0.5 ml.) were added to the residue, and the resulting mixture was agitated at room temperature until all the acetic anhydride had dissolved. The solution was buffered with sodium hydrogen carbonate and extracted with chloroform (5x5 ml.). Evaporation of the organic layer and recrystallisation of the residue from aqueous methanol yielded [\(4-^{3}\text{H}\)] acetanilide (215 mg., 58%, 3.25x10^6 dpm \(^{3}\text{H}\) mmole\(^{-1}\)), m.p. 113-4^\circ (lit. 113-4^\circ).

(d) 4-Bromoacetanilide. A solution of bromine (0.05 ml.) in acetic acid (0.5 ml.) was added at ambient temperature, over a period of 20 min., with vigorous stirring, to a solution of [\(4-^{3}\text{H}\]) acetanilide (64 mg., 3.25x10^6 dpm \(^{3}\text{H}\) mmole\(^{-1}\)) in a acetic acid (0.4 ml.). The resulting mixture was stirred for a further 0.5 h. and poured into water (10 ml.). Excess bromine was reduced with sodium bisulphite, and the solution
was buffered with sodium hydrogen carbonate. Extraction with chloroform (5x5 ml.), evaporation or the organic layer, and recrystallisation of the residue from aqueous ethanol gave 4-bromoacetanilide (80 mg., 79%, 1.1x10⁴ dpm ³H mmole⁻¹), m.p. 167-80° (lit. 77 168°).

(e) In addition to degradation to the above-mentioned derivatives the [3-³H]-precursor was also converted to 2,4,6-tribromo-[3-³H]aniline, as follows. A solution of bromine (0.1 ml.) in acetic acid (0.3 ml.) was added at ambient temperature over a period of 20 min. to a rapidly-stirred solution of [3-³H]aniline (50 mg., derived from [3-³H] benzoic acid, 7.15x10⁷ dpm ³H mmole⁻¹) in acetic acid (1 ml.). The mixture was diluted with water (10 ml.), excess bromine was reduced by addition of sodium bisulphite, and the resulting solution was basified with dilute aqueous sodium hydroxide. Extraction with chloroform (5x5 ml.), evaporation of the organic layer, and recrystallisation of the residue from ethanol, gave 2,4,6-tribromo-[3-³H]aniline (120 mg., 68%, 7.02x10⁷ dpm ³H mmole⁻¹), m.p. 120° (lit. 77 120°).

Growth of Pseudomonas sp. (NCIB 9289, ATCC 11299a)

Cultures of the bacterium were grown in a medium consisting of L-phenylalanine (0.2%), potassium dihydrogen orthophosphate (0.1%), dipotassium hydrogen orthophosphate (0.2%), magnesium sulphate (0.02%), and iron (II) diammonium bis-sulphate (10⁻⁴%) in deionised water at pH 6.5-7.0. The organism was transferred from nutrient slants to portions (10 ml.) of the medium, and
incubated for 24 h. at 30° to facilitate adaption to the use of L-phenylalanine as the sole carbon source. Aliquots (1 ml.) of the resulting cell suspension were used to inoculate quantities (100 ml.) of the medium. These cultures were then grown in a Gallenkamp orbital incubator operated at 150 r.p.m. at 30°. After 36 h. an aqueous solution of labelled phenylalanine (5 ml., typically 45μCi³H and 10μCi¹⁴C) was added. Further incubation for 7 h. resulted in an incorporation of ¹⁴C of ca. 17% into tyrosine, while incubation for 6 h. resulted in ca. 6% incorporation. The relative amounts of radioactive phenylalanine and tyrosine were estimated by radioactive scanning of paper chromatograms of the medium. At the end of the incubation period non-radioactive L-tyrosine (70 mg. or 100 mg.) in 4N-hydrochloric acid was added, and the cells were separated by centrifugation. 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 quickly adjusted to pH 5·5-6·0 with aqueous sodium hydroxide, and the tyrosine was allowed to crystallise slowly. Recrystallisation was repeated several times to ensure constant specific activities of ³H and ¹⁴C, and a single radio spot by paper chromatography.
Degradation of radioactive tyrosine

(a) 4-Methoxycinnamic acid. Tyrosine (250 mg.) was dissolved in 4N-aqueous sodium hydroxide (2 ml.). Dimethyl sulphate (1 ml.) was added, and the mixture was stirred at 0° for 2 h. Sodium hydroxide (0.25 g.) was added, and the temperature was raised to 100° over a period of 0.5 h. After 2 h. at this temperature the reaction mixture was diluted with water (10 ml.) and acidified with hydrochloric acid. Extraction with chloroform (4x5 ml.), evaporation of the solvent, and recrystallisation of the residue from aqueous ethanol gave 4-methoxycinnamic acid (55 mg., 21%), m.p. 173-4° (lit. 175°), τ (DMSO-d6) 0.3 to 0.6 (OH), 2.41 and 3.71 (ABq, 2H, JAB = 16.8Hz), 2.53 and 3.10 (ABq, 4H, JAB = 9Hz), 6.2 (3H, -Oe).

(b) 4-Methoxybenzoic acid. A solution of 4-methoxycinnamic acid (40 mg.) in 10% aqueous sodium carbonate (10 ml.) was treated with 5% aqueous potassium permanganate (10 ml.) at 95° for 12 h. The mixture was cooled, acidified with dilute hydrochloric acid, and extracted with chloroform (5x5 ml.). Evaporation of the organic layer, and recrystallisation of the residue from aqueous acetone gave 4-methoxybenzoic acid (15 mg., 44%), m.p. 183° (lit. 184°), τ (DMSO-d6) 1.4-1.9 (OH), 2.02 and 3.09 (ABq, 4H, JAB = 8.5Hz), 6.17 (OMe).

(c) Treatment of tyrosine with hot hydrochloric acid. Tyrosine (30 mg.) was treated with 4N-hydrochloric acid at 100° for 18 h. to exchange tritium from the 3- and 5- positions. Tyrosine was recovered from the mixture by evaporation of the
TABLE 9

Radioactivities of precursor, tyrosine, and degradation products from *Pseudomonas* feedings

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Total Activity administered (μCi)</th>
<th>Weight of tyrosine (mg.)</th>
<th>Tyrosine 4-Methoxy-cinnamic acid*</th>
<th>4-Methoxy-benzoic acid*</th>
<th>Acid-exchanged tyrosine*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>³H 1⁴C</td>
<td>³H 1⁴C</td>
<td>³H 1⁴C</td>
<td>³H 1⁴C</td>
<td>³H 1⁴C</td>
</tr>
<tr>
<td>DL-[4-³H;carboxy-¹⁴C]-Phenylalanine</td>
<td>42.8 7.89</td>
<td>80 33.7 6.48</td>
<td>32.6 6.26</td>
<td>32.1</td>
<td>- 6.57</td>
</tr>
<tr>
<td></td>
<td>28.4 6.70</td>
<td>100 6.47 1.63</td>
<td>6.30 1.57</td>
<td>6.29</td>
<td>- 1.63</td>
</tr>
<tr>
<td>DL-[4-³H;3,5-²H₂;carboxy-¹⁴C]phenylalanine</td>
<td>42.6 7.24</td>
<td>80 28.7 6.56</td>
<td>27.6 6.24</td>
<td>28.8</td>
<td>- 6.33</td>
</tr>
<tr>
<td></td>
<td>44.1 8.80</td>
<td>100 7.79 2.11</td>
<td>7.69 2.11</td>
<td>8.05</td>
<td>- 2.08</td>
</tr>
</tbody>
</table>

* Corrected for dilution factor
solvent, dissolution of the residue in water (7 ml.), and adjustment of the solution to pH 5.5-6.0 with aqueous sodium hydroxide.

Administration of radioactive precursors to "Texas" daffodils and isolation of norpluvline.

Aqueous solutions of phenylalanine or cinnamic acid, labelled with $^3$H and $^{14}$C, were injected into the hollow flower stalks of blooming "Texas" daffodils. One pot of daffodils (ca. 4 bulbs) was used per feeding. 24 h. later a small quantity of water was fed into each injection point. The plants were harvested 7 days later. In a typical work-up the whole daffodils (260 g.) were cut into small pieces and macerated for 3 days in 1% ethanolic tartaric acid (300 ml.). The resulting mixture was reduced to pulp and the fibrous solid was separated and remacerated for 1 day in ethanolic tartaric acid (250 ml.). The suspension was filtered, the solid washed with ethanol, and the combined filtrates evaporated to ca. 25 ml. in vacuo. The concentrate was acidified with 2N-sulphuric acid (8 ml.), diluted with water to a total volume of 70 ml., and extracted with chloroform (5x20 ml.). The combined extracts were washed with 2N-sulphuric acid (5x10 ml.), the combined aqueous layer was adjusted to pH 8 by addition of sodium hydrogen carbonate, and extracted with chloroform (10x20 ml.). The chloroform extracts were dried, evaporated to a volume of ca. 10 ml., and cooled to 0° overnight. The resulting suspension was filtered and the filtrate was set aside for isolation of
haemanthamine. The solid was dissolved in 2N-sulphuric acid (8 ml.), the solution made alkaline (pH 11) with aqueous sodium hydroxide, and filtered. Carbon dioxide was added to the filtrate to give ca. pH 8, precipitating crude norpluviine (110 mg.), which was recrystallised from methanol to give colourless plates (70 mg.), m.p. 249-50° (lit. 239-41°).

Isolation of haemanthamine. The chloroform-soluble alkaloid fraction from "Texas" daffodils was chromatographed on alumina (activity 3, pH6, 50 g.) packed in benzene. Benzene-ethyl acetate (4:1) eluted at least four alkaloids prior to haemanthamine. Elution with ethyl acetate, evaporation of the solvent, and recrystallisation of the residue from ethyl acetate yielded haemanthamine (typically 28 mg. per pot of 4 bulbs), m.p. 203-5° (lit. 200-1°), m/e 301 (M+, 100%), 286(5%), 272(25%), 269(47%), 268(26%), 257(47%), 240(26%), 227(90%), 225(73%), 211(42%), 181(83%), lit. 78 301(M+, 100%), 286(5%), 272(25%), 269(45%), 268(25%), 257(45%), 240(29%), 227(90%), 225(60%), 211(30%), 181(50%).

Diacetylnorpluviine Norpluviine (20 mg.) was dissolved in pyridine (1 ml.) with gentle warming. Acetic anhydride (0.5 ml.) was added, and the mixture was shaken in the dark for 4 h. at ambient temperature. The resulting solution was poured into water (50 ml.), and sodium hydrogen carbonate was added to give pH > 8. The mixture was extracted with ether (4x10 ml.) and the combined ethereal layer was washed with water (3x5 ml.) The residue obtained by evaporation...
of the ether was filtered through neutral alumina (activity 5) in benzene. Recrystallisation from other-light petroleum (60-80°) gave diacetyl-norpluviine (19 mg., 73%), m.p. 151-2° (lit. 61 150-1°), \(\gamma\) (CDCl₃) 3.16s (1H, arom.), 3.27s (1H arom.), 4.0 br (-O-CH), 4.63br (-C=CH), 5.88, 6.51 (ABq, \(J_{AB} = 14\) Hz, >CH-CH<), 6.28s (OCH₃), 7.26 br (CH₃), 7.50 br (3CH₂), 7.73s (-DOCH₃), 8.11s (-COCH₃).

Formation of haemanthamine picrate, and recovery of the free alkaloid. A saturated solution of picric acid in ethanol (0.4 ml.) was added to a solution of haemanthamine (12 mg.) in ethanol (0.3 ml.) at 20°. The mixture was warmed on a water bath at 70° for 5 min. The solid which separated on cooling was recrystallised from ethanol to give yellow plates of haemanthamine picrate (20 mg., 95%), m.p. 225° (lit. 61 221°). Free haemanthamine was recovered by dissolving the picrate (20 mg.) in water (40 ml.) in the presence of excess sodium carbonate, and warming on a water-bath for 5 min. Extraction with benzene (10 x 10 ml.), filtration of the concentrated extracts through alumina (activity 5), evaporation of the solvent and recrystallisation of the residue from ethyl acetate yielded haemanthamine (8mg., 73%), m.p. 204-5°.

Deuteriation of norpluviine. Norpluviine (86 mg.) was added to a dilute solution of sodium deuterioxide in deuterium oxide (2 ml.). The solution (pH ca. 12) was heated in a sealed tube under nitrogen in the dark at 100°.
for 7 days. The solution was filtered, and \[\text{[8-2H]norpluviine (70 mg.)}\] was recovered from the filtrate by addition of carbon dioxide. Acetylation of the recrystallised product yielded \[\text{[8-2H]norpluviine diacetate, m.p. 150-1^\circ, \gamma (CDCl}_3 3.16s (1H arom.), no signal at } \pi 3.27.\]

**Bromination of \[\text{[8-2H]norpluviine.} \]** \[\text{[8-2H]norpluviine (48 mg.) was dissolved in ethanol (5 ml.) by addition of ethanol hydrogen chloride (5 drops). An ethanolic solution of bromine was added dropwise, and the reaction was followed by t.l.c. (silica; ethyl acetate-methanol, 1:1). When addition of bromine was complete the solution was kept for a further 0.5 h. After evaporation of the solvent the residue was diluted with water, buffered with sodium hydrogen carbonate, and extracted with chloroform (6x5 ml.). The residue obtained by evaporation of the organic phase was treated with acetic anhydride (1 ml.) and pyridine (1 ml.) for 5 h. The resulting solution was diluted with cold water (15 ml.), buffered with sodium hydrogen carbonate, and extracted with ether (3x5 ml.). The residue obtained by evaporation of the organic layer was dissolved in benzene and filtered through alumina (activity 5). Evaporation of the benzene and recrystallisation of the residue from ether-light petroleum (60-80\(^\circ\)) yielded 8-bromonorpluviine diacetate (40 mg., 52\%), m.p. 128-130\(^\circ\) (lit.\(65\) 124-5\(^\circ\), \gamma (CDCl}_3 3.13s (1H, arom.), 4.0br (-OCH), 4.6br (-C=CH), 5.70, 6.63 (ABq, \(J_{AB} = 15H_2\), CH-CH), 6.22s (OCH\(_3\)), 7.23br(CH\(_2\)), \)
7·45br(3CH₂), 7·66s (–COMe), 8·10s (–COMe).

Dilution and degradation of phenylalanine precursors fed to "Texas" daffodils. An aliquot (representing 0·5% of the total quantity of 3H- and 14C-labelled phenylalanine solution fed to a pot of "Texas" daffodils) was added to a solution of non-radioactive phenylalanine (100 mg.) in 5% aqueous sodium carbonate (4 ml.). 5% aqueous potassium permanganate (15 ml.) was added, and the mixture was stirred at 95° for 10 h. After cooling to 20°, treatment with a small quantity of sodium bisulphite and acidification with hydrochloric acid, the mixture was extracted with chloroform (5x5 ml.). Evaporation of the organic layer, sublimation (120°, 0·1 Torr) of the residue, and recrystallisation from water yielded benzoic acid (65 mg., 88%), m.p. 122° (lit. 77 122°), with a labelling pattern corresponding to that of the C₆–C₇ portion of the phenylalanine.
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PART II

Oxygenation of Toluene by Benz[c,d]indazole

1, 2 - Dioxide
INTRODUCTION

In 1970 Whiting and his coworkers, reporting their unsuccessful attempts to obtain \( \text{benz[cd]indazole (I)} \), described the preparation of two derivatives of this compound, the 1-oxide (II), and the 1, 2-dioxide (III). The two oxides arose together during the pyrolysis of 1-azido-8-nitronaphthalene in the gaseous phase, or as a result of the oxidation of 1, 8-diaminonaphthalene with organic peracids, notably \( p \)-nitroperbenzoic acid. The latter procedure proved to be the more satisfactory for large-scale preparations. The higher the peracid to diamine ratio employed, the greater was the ratio of dioxide to monoxide formed. It became apparent, however, that the monoxide was not an intermediate in the formation of the dioxide, since the peracid failed to oxidise the monoxide. It was proposed\(^1\) that the reaction involved formation of 1, 8-dihydroxyaminonaphthalene, which was oxidised to 1-hydroxyamin-8-nitrosonaphthalene (IV). The latter could undergo cyclisation to give \( \text{benz[cd]indazole 1-oxide, or be further oxidised by the peracid to 1, 8-dinitrosonaphthalene, which would cyclise to benz[cd]indazole 1, 2-dioxide (Scheme 1). The relative proportions of the two oxides would thus depend on the relative rates of the intramolecular cyclisation and the intermolecular oxidation of (IV), presumably governed by the concentration and reactivity of the oxidant.} \)
SCHEME 1

(I)

\[
\begin{align*}
&\text{H}_2\text{N} \quad \text{NH}_2 \\
&\text{N} \quad \text{N} \\
&\text{N} = \text{N} \\
\end{align*}
\]

\[
\begin{align*}
&\text{HO-\(\text{NH}\)} \quad \text{HN-\(\text{OH}\)} \\
&\text{N} \quad \text{N} \\
&\text{N} = \text{N} \\
\end{align*}
\]

(II)

(III)

(IV)
Our interest in this work stemmed from the observation that the monoxide could be formed by thermal decomposition of the dioxide, suggesting a loss of atomic oxygen from the latter. If this deoxygenation could be brought about in solution, a useful line of investigation might be to attempt to trap the evolved oxygen species by the use of a suitable acceptor molecule. It is well-known that aromatic amine N-oxides can act as oxygen-atom donors under conditions of photochemical excitation. Investigation of the photo-chemistry of these compounds over the past decade has revealed that intermolecular transfer of atomic oxygen is a common occurrence in solution.

In 1967, Streith et al. reported that irradiation of pyridine N-oxide in benzene resulted in the formation of phenol in 15% yield based on the amount of oxide used. A group of Japanese workers studied the photolysis of a number of aromatic N-oxides, principally 6-methylpyridazine 1-oxide, in a variety of solvents representing alicyclic, olefinic, and aromatic systems. Oxygen transfer was observed in every case. For example, cyclohexane was converted into cyclohexanol; cyclohexene gave a mixture of three products, cyclohexanone (1 part), 1, 2-epoxycyclohexane (5 parts), and a compound believed to be cyclohexan-1,2-diol (1 part); 1-methylocyclohexene gave a mixture of the epoxide and the ketone in a ratio of 4:1; while styrene gave acetophenone (1 part) and styrene oxide (15 parts) (Scheme 2).
Photolysis of 6-methylpyridazine 1-oxide in benzene or naphthalene gave phenols in 30-40% yield based on the weight of oxide consumed.

Thus, in the case of saturated hydrocarbons, it was apparent that an oxygen atom inserted into a C-H bond to give an alcohol. In the case of alkenes, addition of atomic oxygen to C=C to give an epoxide was the prevalent process. It seemed probable that the oxidation of benzene proceeded via formation of benzene oxide (in equilibrium with the valence tautomer oxepin) which underwent rearrangement to phenol. The possibility thus arose that photolysis of aromatic amine N-oxides might provide a chemical model for the action of aryl hydroxylases, and, in particular, might provide an insight into the nature of the "active oxygen" involved in enzymic oxidations. A variety of chemical systems have been investigated over the past twenty years in the hope of finding a useful model for aromatic hydroxylase activity. The discovery of the 1, 2-migration of hydrogen from the position of hydroxylation (the NIH shift) in biological systems has furnished a new criterion of the validity of any proposed model. Absence of the NIH shift when an appropriate substrate is hydroxylated is taken as direct evidence that the action of the model system has no relevance to that of the enzymic system. Hydroxylations accompanied by the NIH shift are regarded as proceeding via addition of an oxygen atom with three pairs of electrons in the valence shell
SCHEME 2

Oxygenation of hydrocarbons by photolysis of 6-methylpyridazine 1-oxide

\[
\begin{array}{c}
\text{O}_{2} + \text{N} \rightarrow \text{O}_{2} + \text{N} \\
\text{Me-} \text{N} \overset{+}{\text{O}} \text{Me} \\
\end{array}
\]

\[
\begin{array}{c}
\text{cyclohexene} \rightarrow \text{cyclohexene-OH} \\
\end{array}
\]

\[
\begin{array}{c}
\text{cyclcopropene} \rightarrow \text{cyclopropene-OH} + \text{cyclohexanone} \\
\end{array}
\]

\[
\begin{array}{c}
\text{cyclohexene-Me} \rightarrow \text{cyclohexene-OH} + \text{cyclohexanone-Me} \\
\end{array}
\]

\[
\begin{array}{c}
\text{styrene} \rightarrow \text{styrene-OH} + \text{benzil-Me} \\
\end{array}
\]

(* major product)
("oxene") to the aromatic substrate to form an arene oxide which rearranges to the corresponding phenol. Hydroxylations which do not show an NIH shift are regarded as proceeding via attack by hydroxyl radicals, or possibly by triplet state "oxene".

Two long-established chemical systems which bring about the hydroxylation of aromatic compounds are Fenton's reagent\(^8\) (ferrous ion and hydrogen peroxide) and Udenfriend's system (ferrous ion, molecular oxygen, and an organic reducing agent such as ascorbate). These, together with the system described by Hamilton\(^10\) (ferric ion, hydrogen peroxide, and a catalytic amount of catechol) failed to produce a significant migration and retention of tritium when \(^{4-\text{H}}\) acetanilide was used as substrate\(^11\). Organic peracids were the first non-enzymic reagents to bring about an NIH shift of deuterium and tritium during the para hydroxylation of benzenoid compounds. When a number of para-deuterated substrates were converted to the corresponding phenols by trifluoroperacetic acid the retention of isotope was similar to that accompanying microsomal hydroxylation. Exceptions were provided by compounds in which the ring was substituted with an electron-releasing group (e.g., anisole), where the retention of isotope was relatively low. It was suggested\(^12\) that the latter effect was due to the high acidity of the peracid medium. As with microsomal hydroxylase activity, hydroxylation by peracids exhibited no detectable primary isotope effect\(^12\) when protium was replaced by deuterium at the position of hydroxylation.
Jerina and his coworkers were the first to carry out investigations into hydroxylation by aromatic amine N-oxides with a view to detecting an NIH shift. Using [4-2H]anisole as substrate it was discovered that deuterium was indeed retained in the 4-hydroxy-derivative when irradiation with a low pressure mercury lamp was carried out for 20 min. at 25° in the presence of the following amine oxides (percentage deuterium retention is given in brackets): pyridazine N-oxide (34), pyridine N-oxide (45), and pyrazine N-oxide (52). Dichloromethane was used as solvent throughout. For comparison, the retention of deuterium upon microsomal hydroxylation of [4-2H]anisole is 60%. In the photolysis experiments 2-hydroxyanisole and 4-hydroxyanisole were formed in a ratio of 1:2. Microsomal hydroxylation of anisole results in a value of 1:10 for the corresponding product ratio. The relatively smaller amount of ortho hydroxylation under biological conditions may indicate that greater steric constraints are operative with the enzymic oxygen-donor than with the amine N-oxide. No 3-hydroxyanisole was detected in either case.

From a photolysis of pyridine N-oxide in the presence of naphthalene in dichloromethane solution 1, 2-epoxy-1, 2-dihydronaphthalene has been isolated in 1/3 yield. Extension of these studies to include para-deuteriated chlorobenzene, bromobenzene, toluene, and acetalanilide revealed that photolysis of pyridine N-oxide brought about hydroxylation of all these substrates with NIH shifts comparable to those resulting
from microsomal activity.

Interpretation of these findings is complicated somewhat by the discovery that the arene oxides derived from benzenoid compounds are photo-isomerised to phenols, and the effect that this process would have on the overall magnitude of deuterium migration and retention is unknown. In contrast, 1,2-epoxy-1,2-dihydronaphthalene is stable under the photo-lysis conditions.

We are unaware of any reported transfer of atomic oxygen from an amine N-oxide to an aromatic substrate in the absence of photochemical excitation, and it was our hope that the thermolysis of benz\[\text{[a]}\]indazole 1,2-dioxide might provide the first example.
DISCUSSION

The object of the present work was to study the reported thermal deoxygenation of benz[cd]indazole 1, 2-dioxide in greater detail. Thermolysis of the dioxide in the liquid phase in the presence of a suitable oxygen-trapping compound might yield valuable information on the proposed loss of oxygen. An ethylenic compound would have been an obvious choice for this purpose, but in view of our interest in aryl hydroxylation we decided to study the thermolysis of the dioxide in an aromatic solvent.

Benz[cd]indazole 1, 2-dioxide was prepared by oxidation of 1, 8-diaminonaphthalene\(^1\) in chloroform with p-nitroperbenzoic acid.\(^{15}\) After separation of p-nitrobenzoic acid, benz[cd]indazole 1-oxide\(^1\) and benz[cd]indazole 1, 2-dioxide\(^1\) were isolated by chromatography on alumina. The solubility of the deep red dioxide in many organic solvents is low; chloroform proved to be one of the best solvents.

We chose toluene as the solvent in which to study the thermolysis of the dioxide for a number of reasons. The use of benzene would have necessitated carrying out the reaction under pressure, since the dioxide is relatively stable in boiling (79°) benzene. In toluene decomposition to the 1-oxido is very slow at 100°, but proceeds at an appreciable rate under vigorous reflux conditions (\(>110°\)). Benz[cd]indazole 1, 2-dioxide dissolves in toluene to the extent of ca. 1 mg. ml.\(^{-1}\) at 80°, a factor which imposes limitations on the scale of the reaction. Our experiments were conducted with
an initial dioxide concentration of 1.25 mg. ml$^{-1}$ in the boiling solvent. The use of toluene as a medium for the thermolysis of the dioxide would be informative as to the mechanism of any intermolecular oxidation which might occur, by permitting a study of the relative importance of ring oxidation and side-chain oxidation, and of the relative abundances of the different isomers resulting from ring oxidation. If measurements of the yields of oxidation products by radiochemical means proved to be desirable, \textit{[methy]l$^{14}$C]toluene was readily available for use as a tracer, and if an NIH shift$^7$ was involved in the formation of a phenolic derivative, methods were available for the synthesis of specimens of toluene labelled at selected ring positions.

A solution of standard concentration of benz [cd$^-$]indazole 1, 2-dioxide in toluene, in the absence of light and oxygen, required a 5 h. period of heating under vigorous reflux conditions to bring about complete decomposition of the starting material. The resulting orange solution contained benz [cd$^-$]indazole 1-oxide in ca. 60% yield, and a number of colourless polar compounds, revealed by t.l.c. Although the solution was apparently free of precipitated material, a black deposit was visible on the walls of the empty reaction flask. Examination of the solution by g.l.c. using a tricresyl phosphate stationary phase indicated that benzaldehyde, benzyl alcohol, and o-cresol were present, in approximate molar yields of 13%, 37% and 11% respectively,
based upon comparison of the peak heights with those of standard solutions. m-Cresol and p-cresol were not chromatographically resolved, but their combined yield was estimated as ca. 5%. Benzoic acid was not detectable using this system. Thermolysis of the dioxide in the absence of special precautions to exclude light and oxygen resulted in formation of the same products in similar yields.

It was possible that the oxidation products, had, in part, arisen by oxygen-donation from benz[1-oxide. To test this, a solution of the 1-oxide (1 mg. ml.⁻¹) in toluene was heated under reflux for 18 h. No measurable amounts of oxidation products were detectable in the resulting mixture.

Isolation of the products from the thermolysis of the dioxide in toluene to establish their identity did not appear to be a very promising approach in view of the small quantities involved. For example, a standard-scale thermolysis would yield less than 2 mg. of the most abundant cresol. It proposed rather to prove the identity of the products and to obtain more accurate estimates of their yields by carrying out the thermolysis of the dioxide in radiolabelled toluene, and isolating the labelled products by isotopic dilution with non-radioactive material. Being at the same time interested in the possibility of detecting an NIH shift, we decided to use toluene labelled in the methyl group with ¹⁴C, and at the para position with tritium.
[4-3H] Toluene was prepared by successive treatment of 4-iodotoluene with lithium metal and [3H] water. (The halotoluene was of identical origin to that employed in the preparation of [4-3H] phenylalanine described in Part I). The [4-3H] toluene was diluted with non-radioactive toluene, purified by fractional distillation, and mixed with [methyl-14C] toluene to give a sample of known 3H/14C ratio. Thermolyses of the dioxide were carried out using standard quantities of radioactive toluene (24 ml.) and benz [cd] indazole 1, 2-dioxide (30 mg.). Two of the oxidation products were isolated from each of three reactions. Firstly, benzyl alcohol and p-cresol were isolated and converted to their 3, 5-dinitrobenzoates for counting. Secondly, benzaldehyde and o-cresol were isolated, and converted to the semicarbazone and the 3, 5-dinitrobenzoate respectively. Thirdly, benzoic acid and m-cresol were isolated, the latter being converted to the 3, 5-dinitrobenzoate for counting.

The isotopic diluent in each case was added to the reaction mixture immediately after the 5 h. reflux period, and the diluted product was separated by chromatography on alumina. The radioactive toluene was first washed off the column with pentane, and fractionally distilled for re-use. Pentane-ether (5:1) eluted benzaldehyde, and ether eluted benzyl alcohol and the three isomeric cresols. The cresols were separated from the benzyl alcohol by washing of the ethereal eluate with dilute aqueous sodium hydroxide. Benzyl alcohol was obtained from the ethereal layer, while acidification of the aqueous layer and re-extraction
with ether gave an ethereal solution of the cresols. Finally, benzoic acid was separated from the alumina by washing with dilute aqueous alkali. The resulting solution was acidified with mineral acid, and the benzoic acid was extracted into chloroform and purified by sublimation.

The benzoic acid, and the derivatives of the other toluene oxidation products, were recrystallised to constant specific radioactivity and $^{3}H/^ {14}C$ ratio (Table 1). The latter process proved to be the most difficult in the case of the isomeric cresols, since all three, if formed in the thermolysis, would be present in the ethereal eluate from each reaction. Purification depended upon separation of the derivative of the isotopically diluted cresol from the derivatives of the other two isomers by repeated crystallisation, and in the case of the 3, 5-dinitrobenzoate of $p$-cresol, a satisfactory result was not obtained, and only an upper limit for the yield of $p$-cresol could be recorded.

Two moles of the dioxide would be required to convert one mole of toluene to benzaldehyde. Hence the yield of the aldehyde based upon the initial amount of dioxide is double the molar yield, i.e. ca. 38%. Summation of the yields of the oxidation products shows that ca. 97% of the oxygen released in forming the 1-oxide can be accounted for. The amount of benzoic acid formed in the reaction can be regarded as negligible since the small amount of this compound detected may have arisen by aerial oxidation of benzaldehyde during the work-up.

It was shown by Daly and his coworkers that microsomal metabolism of toluene resulted in the formation of $o$-cresol,
### Table 1

Radioactivities of derivatives of oxidation products from thermolyses of benz[cd]indazole 1,2-dioxide (1.25 mg.ml⁻¹) in [4-²H; methyl-¹⁴C]toluene (2.37 x 10⁶ dpm.mmole⁻¹; ³H/¹⁴C = 17.52)

<table>
<thead>
<tr>
<th>Oxidation product</th>
<th>Weight of non-radioactive diluent added (mg.)</th>
<th>Derivative</th>
<th>¹⁴C activity of derivative (dpm.mmole⁻¹)</th>
<th>Molar quantity of oxidation product formed</th>
<th>Moles of oxidation product per mole of dioxide(%)</th>
<th>³H/¹⁴C</th>
<th>Retention of ³H(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzyl alcohol</td>
<td>50</td>
<td>Benzyl 3,5-dinitrobenzoate</td>
<td>3.29 x 10⁵</td>
<td>6.41 x 10²</td>
<td>40.0</td>
<td>16.23</td>
<td>92.6</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>56</td>
<td>Benzoic acid</td>
<td>4.03 x 10³</td>
<td>7.8 x 10⁻⁴</td>
<td>0.48</td>
<td>16.24</td>
<td>92.7</td>
</tr>
<tr>
<td>o-Cresol</td>
<td>63</td>
<td>2-Methyl 3,5-dinitrobenzoate</td>
<td>7.40 x 10⁻⁴</td>
<td>1.82 x 10⁻²</td>
<td>11.3</td>
<td>16.32</td>
<td>93.1</td>
</tr>
<tr>
<td>p-Cresol</td>
<td>63</td>
<td>2-Methyl 3,5-dinitrobenzoate</td>
<td>4.89 x 10⁻⁴</td>
<td>6.88 x 10⁻³</td>
<td>3.25</td>
<td>16.23</td>
<td>92.6</td>
</tr>
<tr>
<td>p-Cresol</td>
<td>36</td>
<td>4-Methyl 3,5-dinitrobenzoate</td>
<td>4.89 x 10⁻⁴</td>
<td>6.88 x 10⁻³</td>
<td>4.25</td>
<td>3.5</td>
<td>20</td>
</tr>
</tbody>
</table>
p-cresol, and benzyl alcohol in the molar proportions 1:1:20. No γ-cresol or benzaldehyde were formed. The complete absence of meta hydroxylation is characteristic of the microsomal metabolism of the large majority of monosubstituted benzenes. Notable exceptions are the halobenzenes. The normally-observed pattern of nuclear hydroxylation is taken to show that the oxygen donor of the enzymic hydroxylase system behaves as a weak, selective electrophile. Hence with toluene, where the ortho and para positions are activated toward electrophilic attack by the inductive effect of the methyl group, hydroxylation occurs at these positions to the exclusion of the meta positions. Statistically one might expect ortho hydroxylation to predominate over para hydroxylation by a factor of 2; steric hindrance by the methyl group is regarded as the reason why this is not realised. In our experiments the formation of a substantial quantity of the m-hydroxy derivative (the ratio of γ-cresol to m-cresol being 10:3) strongly suggests that the thermally induced oxygen transfer from benz[αd]indazole 1, 2-dioxide bears little resemblance to the oxygen donation process associated with microsomal hydroxylases.

The latter conclusion is supported when tritium retention in the para hydroxylated product is considered. The data given in Table 1 for p-cresol are based upon the radioactivity of the 3, 5-dinitrobenzoate after eleven recrystallisations. The $^{3}H/^{14}C$ ratio in the ester after the first recrystallisation was ca. half of that in the original toluene, and successive recrystallisations resulted in a decrease in this ratio until,
at the eleventh recrystallisation, the ratio had fallen to 20% of the original, without reaching a stable level. This suggests strongly that the hydroxylation of \([4-3\text{H}]\) toluene at the 4-position had occurred without the 1, 2-migration of tritium which is so characteristic of the corresponding enzymic process. If an NIH shift had occurred, then even if an isotope effect was not operative in the loss of tritium from the keto form of \(p\)-cresol, 50% of the heavy isotope should have been retained in the product.

There remained the possibility that an NIH shift had occurred in the formation of \(p\)-cresol, but that most of the migrated tritium (ortho to the hydroxyl group) had been eliminated by exchange with the medium, either during the thermolysis reaction or during work-up. In order to test this, 4-hydroxy-\([3, 5-3\text{H}_2]\) toluene was prepared by base-catalysed exchange of \(p\)-cresol with tritiated water.\(^{17}\) A sample of this material was treated with 3, 5-dinitrobenzoyl chloride under conditions similar to those employed for the conversion of \(p\)-cresol to 4-methylphenyl 3, 5-dinitrobenzoate. The specific radioactivity of the resulting ester indicated that 98% of the tritium had been retained.

The behaviour of the radiolabels in 4-hydroxy \([3, 5-3\text{H}_2]\) toluene under simulated benz[\text{ad}]indazole 1, 2-dioxide thermolysis conditions was then investigated by adding a sample of the radioactive cresol to a solution of benzyl alcohol, benzaldehyde, \(p\)-cresol, and benz[\text{ad}]indazole 1-oxide in toluene (concentrations corresponding approximately to those found in a thermolysis mixture). After the mixture had been heated for 12 h, the
radioactive cresol was isolated and converted to the 3, 5-
dinitrobenzoate. The specific radioactivity of the ester
indicated that less than 1% of the original tritium had been
eliminated.

We conclude that the conversion of $[{}^{4-3}H]$toluene to
$p$-cresol by benz f$[\text{OD}]$indazole 1, 2-dioxide at temperatures
in the range 110° to 120° is probably not accompanied by the
migration and retention of tritium which is characteristic of
the corresponding enzyme-catalysed process. This seems to
rule out the intermediacy of an arene oxide, but comparison of
our findings with those obtained with other non-enzymic
hydroxylating systems may be of value in formulating a possible
mechanism.

The oxidation of benzene and toluene by Fenton's reagent
has been studied in some detail by Smith and Norman. They
believed that the active agent in bringing about hydroxylation
was the hydroxyl radical, for a number of reasons; e.g. the
isomer ratios obtained in the hydroxylation of nitrobenzene
and chlorobenzene by Fenton's reagent were similar to those
obtained$^{19}$ when the hydroxylating species was produced by the
action of X-rays on water, while the isomer ratios in the
hydroxylation of anisole and fluorobenzene were similar to
those obtained$^{20}$ when hydroxyl radicals were generated by ultra-
violet irradiation of hydrogen peroxide. Intermediacy of the
peroxide radical was ruled out on the following grounds.
Ceric ion reacts with hydrogen peroxide to give the peroxide
radical, which has been identified$^{21,22}$ in the mixture by electron-
spin resonance spectroscopy, but this system is barely

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effective in oxidising benzene; furthermore a mixture contain-
ing titanoons ion and hydrogen peroxide which hydroxylates
chlorobenzene and fluorobenzene, and oxidises benzene to phenol
and biphenyl has been shown to contain hydroxyl radicals but
not peroxide radicals, by the same spectroscopic technique.

The oxidation of toluene by Fenton's reagent resulted in a very great predominance of side-chain oxidation. The
combined yields of benzyl alcohol, benzaldehyde, benzoic acid,
and bibenzyl exceeded the combined yields of the three isomeric
cresols by a factor of 20:1. The molar yields of the cresols
(o : m : p) were in a ratio of 30:2:10. While the ortho :
meta ratio here is greater than that in our benzindazole
dioxide results, the amount of the meta isomer formed is
nevertheless significant when contrasted with the complete
absence of meta hydroxylation in the corresponding enzymic
process. This suggests that meta hydroxylation is a character-
istic feature of radical hydroxylations. The amount of
bibenzyl formed with Fenton's reagent was relatively high,
falling not far short of the total combined yields of benzyl
alcohol and benzaldehyde, and is indicative of the intermediacy
of the benzyl radical in the oxidation of the side-chain. This
is in sharp contrast to our findings with the benzindazole
dioxide, where failure to detect dibenzyl by g.l.c. indicates
that it is formed in less than 1½% yield. It will be noted that
the physical conditions of the dioxide thermolysis differed
greatly from those prevailing in the experiments with Fenton's
reagent. The latter involved a heterogeneous system at ambient
temperature, while the former involved a homogeneous toluene
solution at 110° - 120°.

As we have already noted, the lack of an NIH shift accompanying the para hydroxylation of toluene makes the involvement of an arene oxide intermediate improbable. It seems likely that the active oxygenating species is of a radical character, i.e. atomic oxygen in the triplet spin state. Alternatively, the oxygen transfer may be a bimolecular process, the overall effect of which is equivalent to the transfer of triplet atomic oxygen. In view of our uncertainty on this question, it is reasonable to discuss the process as though free atomic oxygen had been generated. Attack on the aromatic nucleus would proceed via a diradical intermediate with the unpaired electrons in parallel spin states, thus precluding the formation of an arene oxide (Scheme 3). The triplet atomic oxygen would bring about benzylic oxidation by a non-concerted mechanism involving abstraction of a hydrogen radical (Scheme 4). Further radical oxidation would lead to the formation of benzaldehyde, while dimerisation of the benzyl radical would give bibenzyl. The absence of a substantial yield of this compound suggests that the dimerisation process is less favoured under the thermolysis conditions than under the conditions of the Fenton's reagent experiments.

The large yield of benzaldehyde in the thermolysis indicates that the oxidation of benzyl alcohol proceeds very readily, while the virtual absence of benzoic acid shows that the oxidation of the side-chain does not proceed further. No products were detected in which both the side-chain and the
SCHEME 3

\[
\text{Me} \quad + \quad \bullet \quad \rightarrow \quad \text{Me} \quad + \quad \bullet \quad \rightarrow \quad \text{Me}
\]

SCHEME 4

\[
\text{H}_2\text{C} = \text{H} \quad \rightarrow \quad \text{H}_2\text{C} \quad + \quad \bullet \text{OH} \quad \rightarrow \quad \text{H}_2\text{C} - \text{OH}
\]

110.
It will be noted from Table 2 that for each of the oxidation products (p-cresol excepted) there is an apparent decrease in the $^3$H/$^{14}$C ratio of ca. 7% relative to the original toluene. This may indicate a loss of tritium by exchange from the para position under the conditions of the thermolysis, but perhaps a more probable explanation is that there was an error in the measurement of the specific radioactivity of the toluene, where recrystallisation to constant specific activity is, of course, not possible.

A logical extension of these investigations would be to study the behaviour of benz[cd]indazole 1, 2-dioxide under photolytic conditions. There are good reasons for expecting a loss of atomic oxygen similar to that observed with other aromatic amine N-oxides. In the presence of a suitable substrate, a comparison of the photolytic product distribution with that obtained under thermolytic conditions might be informative as to the nature of the active oxygen species.
EXPERIMENTAL

The general procedures were those described in Part I, with the following additions. Ultraviolet spectra were recorded using a Unicam SP 8000 spectrophotometer. Gas-liquid chromatography was carried out on a Pye Series 104 instrument with a hydrogen flame ionisation detector, using a 5 ft. column packed with 10% trioresyl phosphate supported on chromosorb W, operated at 160°C.

**p-Nitroperbenzoic acid**  Sodium peroxide (10.3 g.) was added to dry tetrahydrofuran (40 ml.) under anhydrous conditions, and the resulting suspension was stirred vigorously at -20°C. A solution of p-nitrobenzoyl chloride (3.7 g.) in tetrahydrofuran (10 ml.) was added slowly, followed by ice (2 g.). The temperature was maintained within the range -15°C to -5°C throughout. A further solution of p-nitrobenzoyl chloride (14.8 g.) in tetrahydrofuran (40 ml.) was added over a period of 0.5 h., followed by gradual addition of ice-cold water (200 ml.). After being stirred for a further 0.5 h. (temperature below 0°C) the mixture was extracted with chloroform (3 x 70 ml., at 0°C), and the aqueous solution was added slowly to 20% sulphuric acid (75 ml., at 0°C). The resulting mixture was extracted with ether (400 ml. + 100 ml., at 0°C). The ethereal solution was washed with water (50 ml.), and with phosphate buffer (0.25M in Na₂HPO₄ and KH₂PO₄, 200 ml. + 200 ml. + 100 ml., at 0°C), dried (Na₂SO₄), and concentrated to a volume of ca. 100 ml. in vacuo. Gradual addition of light petroleum (b.p. 60-80°C, 200 ml.) induced crystallisation of p-nitroperbenzoic acid.
(11 g., 75%), m.p. (sealed tube) 134° (lit. 23 136-7°).
Assay (titration against standard sodium thiosulphate solution in the presence of potassium iodide) 79%.

Oxidation of 1, 8-diaminonaphthalene with p-nitrobenzoic acid

1, 8-Diaminonaphthalene (5 g., purified by Soxhlet extraction with light petroleum (b.p. 40-60°) and recrystallisation from benzene-light petroleum) was added to a suspension of p-nitrobenzoic acid (40 g.) in chloroform (1600 ml.), and the resulting mixture was stirred at ambient temperature for 2.5 h. The reaction was followed by t.l.c. (alumina; benzene-chloroform, 1:1). The resulting mixture was filtered, and the filtrate was washed with dilute aqueous sodium carbonate until the washings were alkaline, dried, and concentrated to a low volume. The concentrated chloroform solution was chromatographed on alumina (activity 3, 800 g.) packed in carbon tetrachloride. Benzene-ether (9:1) eluted a mixture of four compounds, which on crystallisation from benzene-light petroleum (b.p. 40-60°) gave the intense orange benz[cd]indazole 1-oxide (0.8 g., 15%), m.p. 157-8° (lit. 1 156-7°), \( \lambda_{\text{max}} \) 1492s, 1468s, 1454s, and 1442m. Elution with chloroform and recrystallisation of the eluted material from chloroform-light petroleum (b.p. 40-60°) gave the purple benz[cd]indazole 1, 2-dioxide (1.6 g., 27%), m.p. (sealed tube) 185° (lit. 1 187°), \( \lambda_{\text{max}} \) (CHCl₃) 246, 305, 355, 377, 397, and 510 (logε 4.36, 3.74, 3.96, 3.99, 3.88, and 3.04). (The literature 1 quoted a \( \lambda_{\text{max}} \) at 335, rather than 355). \( \lambda_{\text{max}} \) 1490s, 1470s, and 1434s.

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Thermolysis of benz[cd]indazole 1, 2-dioxide in toluene.

A solution of benz[cd]indazole 1, 2-dioxide in oxygen-free toluene (1.25 mg. ml\(^{-1}\)) was heated vigorously under reflux in the dark in an atmosphere of nitrogen. Examination by t.l.c. (alumina, benzene-chloroform, 1:1) showed total decomposition of the dioxide in 5 h., benz[cd]indazole 1-oxide being the principal product. Examination by g.l.c. indicated the following products (retention times in min.): benzaldehyde (1.50), benzyl alcohol (2.95), o-cresol (5.4), m- and p-cresol (not resolved, 6.9). The system was not suitable for the detection of benzoic acid (retention time 21 min.). Estimated molar yields based on the dioxide: benzaldehyde (12\%), benzyl alcohol (37\%), o-cresol (11\%), m- and p-cresol (5-3\% combined).

Effect of benz[cd]indazole 1-oxide on boiling toluene.

A solution of benz[cd]indazole 1-oxide in toluene (1 mg. ml\(^{-1}\)) was heated under reflux for 18 h. G.l.c. revealed no measurable amounts of oxidation products.

\(^{[4-3\text{H}]\text{Toluene}}\). 4-Iodotoluene (sublimed, 2.18 g.) in dry ether (30 ml.) was stirred with lithium (0.2 g.) under nitrogen for 3 h. Tritiated water (0.2 ml., 3.6 mCi/mole\(^{-1}\)) was added, followed, 0.3 h. later, by an excess of water. The ethereal layer was dried (Na\(_2\)SO\(_4\)) and toluene (30 ml.) was added. Fractional distillation yielded \(^{[4-3\text{H}]\text{Toluene}}\) (26 ml., 3.1x10\(^2\) mCi ml\(^{-1}\)).
Thermolysis of benz[cd]indazole 1, 2-dioxide in $[^{4-3H; methyl-14C}]$toluene. $[^{4-3H}]$Toluene (ca. 4,200 Ci) and $[^{methyl-14C}]$toluene (ca. 240 u Ci, Radiochemical Centre, Amersham) were mixed and isotopically diluted with non-radioactive toluene to give a total volume of 24 ml. ($^{3H/^{14C}} = 17.54$). (An aliquot, isotopically diluted by a factor of 10$^3$ and filtered through alumina (activity 1), had $^{3H/^{14C}} = 17.52$). Benz[cd]indazole 1, 2-dioxide (30 mg.) was dissolved in the doubly-labelled toluene (24 ml.) and heated under vigorous reflux for 5 h. T.I.C. showed complete decomposition of the dioxide. The work-up procedure of the resulting solution varied according to the reaction product to be isolated.

(a) o-, m-, and p-Cresols. One of the three isomers was isolated from each of three successive thermolyses. The non-radioactive cresol (typically 40 mg.) was added to the reaction mixture, and the resulting solution was chromatographed on alumina (activity 1, 30 g.) packed in pentane. Pentane (100 ml.) eluted $[^{4-3H; methyl-14C}]$toluene which was fractionally distilled for re-use. Ether (150 ml.) eluted the oxygenated toluene derivatives. The ethereal solution was washed with $\mathrm{N-NaOH}$ (5x8 ml.). The aqueous phase was acidified with hydrochloric acid and extracted with ether (6x5 ml.). The ethereal layer was dried and concentrated in vacuo to a volume of ca. 1 ml. The residue was treated with pyridine (1 ml.) and 3, 5-dinitrobenzoyl chloride (150 mg.) and heated under reflux for 50 min. 2N-Hydrochloric acid (10 ml.) was added, and the
resulting mixture was cooled in ice for 10 min. The precipitate was collected, washed with water, and stirred with N-sodium hydroxide (5 ml.). The undissolved material was separated, dried, and filtered through alumina (activity 3) in benzene. Evaporation of the eluate and recrystallisation of the residue from chloroform-light petroleum (b.p. 60-80°) yielded the 3, 5-dinitrobenzoate of the labelled cresol (typically 70 mg.), characterised as follows: 2-methylphenyl 3, 5-dinitrobenzoate (from o-cresol), m.p. 138-9° (lit. 138°); 3-methylphenyl 3, 5-dinitrobenzoate (from m-cresol), m.p. 164-5° (lit. 165°); 4-methylphenyl 3, 5-dinitrobenzoate (from p-cresol), m.p. 188-190° (lit. 189°).

(b) Benzyl alcohol. Non-radioactive benzyl alcohol (50 mg.) was added to the reaction mixture. Chromatography was carried out as before. The ethereal eluate, after being washed with N-sodium hydroxide, was dried and concentrated to ca. 1 ml. The residue was treated with pyridine (1 ml.) and 3, 5-dinitrobenzoyl chloride (200 mg.) and heated under reflux for 50 min. Work-up of the resulting mixture by a similar procedure to that employed for the cresol derivatives yielded [4-3H; methylene-14C] benzyl 3, 5-dinitrobenzoate (80 mg.), m.p. 114° (lit. 113°).

(c) Benzaldehyde. Non-radioactive benzaldehyde (63 mg.) was added to the reaction mixture, and the resulting solution was chromatographed on alumina (activity 3, 30 g.) packed in pentane. Pentane eluted radioactive toluene which was recovered as before. Pentane-ether (5:1, 50 ml.) eluted benzaldehyde.

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The latter solution was concentrated to ca. 2 ml., and semi-carbazide hydrochloride (110 mg.), sodium acetate (140 mg.), and water (2 ml.) were added to the residue. The mixture was shaken vigorously for 2 h. The precipitate thus formed was separated and recrystallised from methanol to yield $\left[4^{-3}H\right]$ benzaldehyde $\left[\text{imin0-}^{14}\text{C}\right]$ semicarbazone (105 mg.), m.p. 224° (lit. 24 224°).

(a) Benzoic acid. Non-radioactive benzoic acid (56 mg.) was added to the reaction mixture. Chromatography was carried out as before. After exhaustive elution with ether the alumina was stirred with water (50 ml.), and dilute aqueous sodium hydroxide was added to give a pH of ca. 9. The alumina was separated, and the filtrate was acidified dilute hydrochloric acid and extracted with chloroform (5x5 ml.). The organic layer was dried, the solvent was removed in vacuo, and the residue was sublimed and recrystallised from water to yield $\left[4^{-3}H;\text{carboxyl-}^{14}\text{C}\right]$ benzoic acid (46 mg.), m.p. 122° (lit. 24 122°).

4-Hydroxy-$\left[3,5^{-3}H_2\right]$ toluene. p-Cresol (5.4 g., 50 mmole), triethylamine (5.05 g., 50 mmole), and tritiated water (3.6 mCi.mmole$^{-1}$, 0.1 ml.) were heated together at 100-105° for 2 h. Water (50 ml.) was added, and the resulting solution was acidified with hydrochloric acid and extracted with ether (2x20 ml.). The organic layer was dried ($\text{Na}_2\text{SO}_4$) and fractionally distilled to yield 4-hydroxy $\left[3,5^{-3}H_2\right]$ toluene (4.8 g., ca. 10$^8$ dpm.mmole$^{-1}$).
Retention of tritium in 4-methyl [2,6-\(^3\)H\(_2\)]phenyl 3, 5-
dinitrobenzoate derived from 4-hydroxy-[3,5-\(^3\)H\(_2\)]toluene
4-Hydroxy[3,5-\(^3\)H\(_2\)]toluene (60 mg., 1.103x10\(^7\) dpm.mmole\(^{-1}\)),
3, 5-dinitrobenzoyl chloride (250 mg.), and pyridine (1 ml.)
were heated under reflux for 1 h. Work-up by the procedure
outlined above yielded 4-methyl [2,6-\(^3\)H\(_2\)]phenyl 3, 5-
dinitrobenzoate (150 mg., 1.085x10\(^7\) dpm.mmole\(^{-1}\) = 98.4\% 
retention of radiolabel).

Retention of tritium in 4-hydroxy-[3,5-\(^2\)H\(_2\)]toluene under
simulated benz [cd]indazole 1, 2-dioxide thermolysis conditions
A solution of \(\epsilon\)-cresol (0.08 mg. ml\(^{-1}\)), benzyl alcohol
(0.25 mg. ml\(^{-1}\)), benzaldehyde (0.086 mg. ml\(^{-1}\)),
benz [cd] indazole 1-oxide (1mg.ml\(^{-1}\)), and 4-hydroxy-
[3-\(^3\)H]toluene (19 mg., 1.888x10\(^6\) dpm.mmole\(^{-1}\) in toluene
was heated under reflux for 12 h. 4-Hydroxy-[3,5-\(^3\)H\(_2\)]-
toluene was isolated by the normal procedure for cresols (vide
supra), and treated with 3, 5-dinitrobenzoyl chloride to yield
4-methyl [2,6-\(^3\)H\(_2\)]phenyl 3, 5-dinitrobenzoate, 1.644x10\(^6\)
dpm.mmole\(^{-1}\) (87\% retention of radiolabel).

Stability of oxygenated reaction products under simulated
benz [cd]indazole 1, 2-dioxide thermolysis conditions.
A solution of \(\epsilon\)-cresol (0.04 mg. ml\(^{-1}\)), \(\epsilon\)-cresol (0.08 mg.
ml\(^{-1}\)), benzyl alcohol (0.25 mg. ml\(^{-1}\)), benzaldehyde (0.086 mg.
ml\(^{-1}\)), and benz [cd] indazole 1-oxide (1mg. ml\(^{-1}\)) in toluene
was heated under reflux. Samples withdrawn at intervals up to
12.5 h. showed a constant concentration of the oxygenated
toluene derivatives by g.l.c.
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