STUDIES OF REDUCED PTERINS AND RELATED COMPOUNDS

A thesis submitted to the Australian National University for the degree of Doctor of Philosophy by Henning Schou

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May, 1978
CERTIFICATE OF ORIGINALITY

The work described in this thesis was carried out by the candidate at The Australian National University. Where the work of others was employed or quoted, appropriate references have been included.

Hunng Schae
ACKNOWLEDGEMENTS

I express my deepest gratitude to Dr W.L.F. Armarego for his supervision throughout this project, for his patience and encouragement during the more difficult and less fruitful periods and for his innumerable suggestions which make up the backbone of this thesis.

I sincerely thank Dr D.J. Brown and the entire staff of the Medical Chemistry Group, Drs J.H. Lister and D.D. Perrin in particular, for helpful discussions and assistance.

I am indebted to Dr A.J. Jones at the National NMR Centre for help in connection with the $^{13}$C n.m.r. spectra.

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My wife, Judy, deserves special thanks for her understanding, tolerance and great help during my period of writing.

Finally, acknowledgement is made for the award of an Australian National University Scholarship.

H.S.
SUMMARY

The present work seeks to contribute to the elucidation of the absolute configurations at C(6) in the enantiomers of 6-methyl-5,6,7,8-tetrahydropterin and, in general, in reduced pterins which are natural cofactors, e.g. 5,6,7,8-tetrahydrobiopterin and tetrahydrofolic acid.

The relative stereochemistry of hydrogen addition to C(6) and C(7) in 6-methyl-, 6,7-dimethylpterin and in 2,4-diamino-6-methylpteridine is deduced from an analysis of the relatively simple p.m.r. spectra of deuterated derivatives such as 7-deutero-6-methyl-, 6-trideuteromethyl-, 7-deutero-6-trideuteromethyl- and 6,7-bis(trideuteromethyl)-5,6,7,8-tetrahydropterins, and of 2,4-diamino-7-deutero-6-trideuteromethyl(partial)-5,6,7,8-tetrahydropteridine. An account of the synthesis of the deuterated derivatives is given. The catalytic (PtO₂) addition of two molecules of hydrogen to 7-deutero-6-trideuteromethylpterin yields a 0.8:1 mixture of cis- and trans-7-deutero-6-trideuteromethyl-5,6,7,8-tetrahydropterin. A similar reduction of 2,4-diamino-7-deutero-6-trideuteromethyl(partial)pteridine gives a 1:1 mixture of cis- and trans-2,4-diamino-7-deutero-6-trideuteromethyl(partial)-5,6,7,8-tetrahydropteridine. On the other hand, catalytic reduction of 6,7-dimethylpterin is stereospecific, as revealed by the p.m.r. spectrum of
6,7-bis(trideuteromethyl)-5,6,7,8-tetrahydropterin, and forms only the cis-5,6,7,8-tetrahydro derivative. Reduction of 6,7-dimethylpterin with sodium in ethanol provides a 1:1 mixture of cis- and trans-6,7-dimethyl-5,6,7,8-tetrahydropterin.

Optical resolution of racemic 6-methyl-5,6,7,8-tetrahydropterin is achieved via recrystallization of the diastereomeric 2S,3S-(-)-O,O'-dibenzoyltartaric salts.

Thorough methylation of 5,6-dimethyl-5,6,7,8-tetrahydropterin by methyl iodide in methanol containing one molar excess of sodium hydroxide furnishes 1,3,5,6-tetramethyl-5,6,7,8-tetrahydropterinium chloride. The latter salt is shown by p.m.r. and $^{13}$C n.m.r. spectroscopy to be identical with the tetramethyl-5,6,7,8-tetrahydropterinium chloride obtained through methylation of 6-methyl-5,6,7,8-tetrahydropterin. The assignment of the p.m.r. signals from CH$_3$(1), CH$_3$(3) and CH$_3$(5) in the spectrum of 1,3,5,6-tetramethyl-5,6,7,8-tetrahydropterinium chloride is deduced from the p.m.r. spectra of the products obtained after trideuteromethylation of 5,6-dimethyl-, 3,5,6-trimethyl- and 1,5,6-trimethyl-5,6,7,8-tetrahydropterins in methanol containing one molar excess of sodium hydroxide. The study of methylation by trideuteromethyl iodide revealed further that methylation of 5,6-dimethyl-5,6,7,8-tetrahydropterin probably takes place via intermolecular methylation by the quaternary 5,5-dimethyl salt.

The 5-methyl group in 1,3,5,6-tetramethyl-5,6,7,8-tetrahydropterinium chloride is lost when the salt is
treated with Dowex 50W/3\textsubscript{N}-aqueous ammonia, 3\textsubscript{N}-aqueous ammonia or N-sodium hydroxide. This demethylation and the exchange of the 5-methyl group in 5,6-dimethyl-5,6,7,8-tetrahydropterin during methylation allow a mechanism to be proposed for the enzymatic transfer of the 5-methyl group in 5-methyl-5,6,7,8-tetrahydrofolic acid during biological methylations.

1,3,6-Trimethyl- and 1,3,5,6-tetramethyl- 5,6,7,8-tetrahydropterinium chloride hydrochlorides and 1,3,6-trimethyl-2-methylamino-4(3\textsubscript{H})-oxo-5,6,7,8-tetrahydropyridinium chloride hydrochloride are found to be considerably more stable towards aerial oxidation than 6-methyl-, 1,6-dimethyl-, 5,6-dimethyl-, 6,7-dimethyl- and 6,8-dimethyl- 5,6,7,8-tetrahydropterin hydrochlorides and 6-methyl-2-methylamino-4(3\textsubscript{H})-oxo-5,6,7,8-tetrahydropteridine hydrochloride.

Due to the stability of 5,6,7,8-tetrahydropyridins enhanced by methylation, it should be possible to degrade one of the methylated enantiomers of 6-methyl-5,6,7,8-tetrahydropterin without loss of the chiral centre at C(6). The possible degradation product could then be related to chiral 1,2,4-trimethylpiperazine. The synthesis of the 2S-(+)-1,2,4-trimethylpiperazine hydrochloride from glycyl-S-alanine, using reactions which do not affect the asymmetric centre at C(2), is described.
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1.1 General description of naturally occurring pteridines

1.1.1 Early history of naturally occurring pterins and pteridines (excluding folic acid)

The history of pteridines began in 1889 when F. Gowland Hopkins initiated investigations of extracts of pigments from butterfly wings and tried to determine their nature. This led to the isolation of a yellow compound from the brimstone butterfly and six years later to the isolation of a white pigment from the cabbage butterfly. For the next thirty years no further studies were made. In 1925 Schöpf continued the study in the laboratories of H. Wieland. Schöpf and Wieland extracted the same pigments (from some 200,000 butterflies) and managed to purify them to a higher degree. However, elucidation of the chemical structure of these pigments was delayed due to (a) lack of melting point (thus removing an important criterion for purity), (b) poor solubility in all common solvents (hence recrystallization was very difficult), (c) resistance to complete combustion (which led to erroneous analytical results, indicating low nitrogen content), (d) the misleading fact that pterins upon degradation give substances similar to those obtained from the degradation of purines, and (e) the fact that no molecular weights could be obtained. These pigments were called xanthopterin (1.1) and leucopterin (1.2)
It was in 1964 that Purrmann, with Weingart, showed that the two pigments were structurally similar to isoxanthopterin (1.3). He named the pigment called isoxanthopterin (1.3). He named this pigment xanthopterin by heating 2,4,5-triaminopyrimidine-6(3H)-one with oxalic acid (1.2) and not xanthopterin from the same pyrimidine and dichromate reaction (1.1) (1.4) by oxidizing the benzene ring to a dicarboxylic acid (1.5) and decarboxylating this to the dihydropteridine (1.6), which he called "alloxazine". This was an unfortunate name, since it is now commonly used for the benzo derivatives of pteridine. The name was later changed to "lumazine" (1.6) (1.7) with cobalt to give 5,6-diphenylpteridine (1.9).

Two years earlier in 1966, Kühn and Meyerhöfer showed that this was a very good synthesis by starting with the pteridines based on pyrimidine. A third method of the preparation of lumazine was published in 1937 by Kühn and Petry (21).
because of the colour (Gk. ξανθός, xanthos = yellow; λευκός, leukos = white) and source (Gk. πτερόν, pterin = wing).

It was not before 1940 that Purrmann together with Wieland showed that the two pigments were amino-oxy derivatives of tetraazanaphthalene. At the same time he also elucidated the structure of a related pigment called isoxanthopterin (1.3). Purrmann synthesized leucopterin by heating 2,4,5-triaminopyrimidin-6(1H)-one with oxalic acid, and made xanthopterin from the same pyrimidine and dichloroacetic acid.

It then turned out that such derivatives had been made by Kühling as early as 1894 in attempts to obtain chromophores of tolualloxazine (1.4) by oxidizing the benzene ring to a dicarboxylic acid (1.5) and decarboxylating this to the dioxopteridine (1.6), which he called "alloxazine". This was an unfortunate name, since it is now commonly used for the benzo derivatives of pteridine. The name was later changed to 'lumazine' (1.6). Gabriel and Sonn prepared lumazine by treating pyrazine-2,3-dicarboxamide (1.7) with potassium hypobromite.

The previous year, in 1906, Isay had published the first example of a general reaction for the synthesis of pteridines, namely the condensation of 4,5-diaminopyrimidine (1.8) with benzil to give 6,7-diphenylpteridine (1.9). Two years later Sachs and Meyerheim had shown that this was a very good synthesis by making ten more pteridines based on Isay's example. A third method for the preparation of lumazine was published in 1937 by Kuhn and Cook.
Isay's Pteridine Synthesis

(1.10)
It involved the reaction of 4,5-diamino-2,6-dioxopyrimidine (1.10) and glyoxal; and lumazine (1.6) was recognized as a degradation product of riboflavin.

1.1.2 Nomenclature of pteridines (excluding folic acid)*

H. Wieland coined the term 'pteridine' for the tetraazanaphthalene system in 1941. This system is called pyrimido[4.5-b]pyrazine in the first edition of 'The Ring Index', but in the second edition (1960) the name was altered to pyrazino[2,3-d]pyrimidine. The numbering for pteridine shown in formula 1.11 was approved by IUPAC. It was proposed by Kuhn and Cook in 1937 and is consistent with the rules normally applied to aza derivatives of naphthalene. Unfortunately another system (see formula 1.12) designed to relate pteridine to purine has been used in early publications from European (particularly German) laboratories. This numbering, however, has fallen out of usage. The terms 'pterin' and 'pteridine' have caused considerable confusion. 'Pterin' was originally used as a collective term for the butterfly wing pigments and also, occasionally, as an abbreviation for pteridine. Pfleiderer suggested that use of the term 'pterin' should be restricted to include only derivatives of 2-amino-4-hydroxypteridine. This term is adopted in the succeeding chapters.

* For further reading see references 22, 23.
Many of the known pteridine derivatives contain one or more hydroxy groups which are aliphatic to the ring nitrogen atoms and the latter is possible (e.g., 1.13 = 1.14). It has been customary in the biochemical literature to name these compounds as hydroxy-derivatives, although physical data (ultraviolet spectra and pK values) indicate that — from an equilibrium point of view — there is a considerable equilibrium between these tautomeric forms, largely in favour of the lactam form.

(Numbering system for pteridine approved by IUPAC)

\[
\begin{array}{c}
\text{OH} \\
N \text{N} \\
\text{O} \\
N \text{N}
\end{array}
\]

(1.12)

Not recommended numbering

The revelation of a new naturally occurring heterocyclic ring system in the pteridine field tremendously, and it was shown that these compounds were widely distributed in nature although usually in very low concentrations. Their conspicuous fluorescence made it possible by the then newly developed technique of chromatography to detect, isolate, and characterize amino acids in fish, blood, stomach mucus and excreta (see Table XI for a few examples).

\[
\begin{array}{c}
\text{OH} \\
\text{OH} \\
N \text{N} \\
\text{OH}
\end{array} \quad \Leftrightarrow \quad \begin{array}{c}
\text{HN} \\
\text{N} \\
\text{O} \\
\text{N}
\end{array}
\]

(1.13) (1.14)
Many of the known pteridine derivatives contain one or more hydroxy groups which are alpha to the ring nitrogen atoms and tautomerism is possible (e.g. $1.13 \Leftrightarrow 1.14$; lactim $\Leftrightarrow$ lactam). It has been customary in the biochemical literature to name these compounds as hydroxy derivatives although physical data (ultraviolet spectra and $pK_a$ values) indicate that – from an energy point of view – the equilibrium between these tautomeric forms lies largely in favour of the lactam form. 27

1.1.3 Further history of discoveries and syntheses of some naturally occurring pterins and pteridines (excluding folic acid)

The revelation of a new naturally occurring heterocyclic ring system stimulated research in the pteridine field tremendously, and it was shown that these compounds were widely distributed in nature although usually in very low concentrations. Their conspicuous fluorescence made it possible by the then newly discovered technique of paper chromatography to detect, isolate, and characterize minute amounts in fish, silkworms, amphibians, and especially in insects (see Table 1.1 for a few examples).
Several reviews, which discuss the pteridines found especially in insects,29 in microbes,30 and in amphibia and fish,31 are available.

In the following, attention will be directed to a few representative pteridines which will serve as prototypes for the methods that have been used for structure assignment and synthesis of the group as a whole.

<table>
<thead>
<tr>
<th>Pterin</th>
<th>Butter-flies</th>
<th>Fruit-flies</th>
<th>Blow-fly</th>
<th>Honey-Bee</th>
<th>Skin of Amphibian</th>
<th>Skin of Fish</th>
<th>Skin of Reptiles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pterin-6-COOH</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Xanthopterin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Isoxanthopterin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Leucopterin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Biopterin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Sepiapterin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Isosepiapterin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
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<tr>
<td>Drosopterins</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
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<td>+</td>
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<td>+</td>
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<tr>
<td>Lepidopterin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Pterorhodin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Isoxanthopterin-6-COOH</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Ichthyopterin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td></td>
</tr>
</tbody>
</table>
Pterorhodin (1.15) was discovered in 1944. It is a violet substance which results from autoxidation of acid solutions of crude butterfly wing pigments. On oxidation, it gives xanthopterin-7-carboxylic acid (1.16) and leucopterin (1.2).

Erythropterin (1.17) is an orange-red pigment which was first isolated from a South African butterfly by Schöpf and Becher\textsuperscript{34} in 1936. The correct structure was deduced by Pfleiderer\textsuperscript{35} who obtained it chromatographically pure in 1962. Hydrolysis of erythropterin gave oxalic acid and 7-methylxanthopterin (1.18), confirming the presence of the pyruvate side-chain. The structure 1.17 is likely to exist in a tautomeric form, most probably the enamine (1.19), and was confirmed by unambiguous synthesis.

Once the correct structure for erythropterin was obtained, the structures of lepidopterin (1.20) and ekapterin (1.21) were established by Viscontini and Stierlin\textsuperscript{36} by means of the interconversions shown in Scheme 1.1.

Urothione (1.22) is a sulphur-containing pteridine found in human urine in 1940 by Koschara,\textsuperscript{37} who estimated its empirical formula as $\text{C}_{11}\text{H}_{13}\text{O}_3\text{N}_5\text{S}_2$. The correct structure (1.22) was obtained by Tschesche, Korte and Heuschkel\textsuperscript{38} fifteen years later. It is also present in the liver of man and cattle.

Biopterin (1.23) is another pterin which was first isolated from human urine.\textsuperscript{39} It appeared to be widely distributed in nature (see Pfleiderer's review\textsuperscript{26}).
\begin{align}
(1.15) & \quad \rightarrow [\text{O}] \\
(1.16) & + \\
(1.17) & + \\
(1.18) & + \text{COOH} \quad \text{COOH} \\
(1.19) & 
\end{align}
Erythropterin (1.17) $\overset{\text{NH}_3\text{aq.}}{\rightleftharpoons} \overset{\text{H}^+}{\text{H}}$ Lepidopterin (1.20)

$\text{NaBH}_4$

$\overset{\text{O}_2\text{air}}{\text{H}}$ Racemic ekapterin (1.21)

Scheme 1.1
The structure of the compound was shown by synthesis to be 6-D-arabino-5,2'-dihydroxypropyllyterin.\footnote{40,41} Concerning the stereochemistry of bioppterin, see Section 1.8, p. 28.

The condensation of 2,4,6-tri-iodo-1-chloropyrimidine gave a 7,8-endo-norbioppterin (1.25) which was identical with the substance isolated from the leaves of A. angustifolium.\footnote{42} The 6- and 7-isomers of 2,4,6-tri-iodo-1-chloropyrimidine can be separated by partition chromatography on a silica gel column.\footnote{43} The 6- and 7-isomers of 2,4,6-tri-iodo-1-chloropyrimidine have different biological activities.\footnote{44,45} The 7-isomer is the more active.

The synthesis of bioppterin can be carried out in a similar way.\footnote{46,47} The 6- and 7-isomers of bioppterin can be separated by partition chromatography on a silica gel column.\footnote{48} The 6- and 7-isomers of bioppterin have different biological activities.\footnote{49,50} The 7-isomer is the more active.

A derivative of bioppterin occurs in the eyes and skin of insects, and in the fruit of apple and in the urine of man.\footnote{51} A glucoside of bioppterin has been isolated from the blue gland of the lepidoptera.\footnote{52} The structure of the compound was determined by synthesis from 2-acetylisouoxanthopterin.\footnote{53} However, until the hypothesized D-arabino configuration of the bioppterin was first isolated in 1943 from carp, it is not known whether bioppterin resembles isouoxanthopterin.\footnote{54,55} The structure of the compound was determined by synthesis from 2-acetylisouoxanthopterin.\footnote{56} However, until the hypothesized D-arabino configuration of the bioppterin was first isolated in 1943 from carp, it is not known whether bioppterin resembles isouoxanthopterin.\footnote{57}
The structure of the compound was shown by synthesis to be 6-\((S\)-erythro-1,2-dihydroxypropyl\)pterin\(^{40,41}\) (concerning the stereochemistry of biopterin see Section 1.2, p.25).

The condensation of 2,4,5-triaminopyrimidin-6(1\(H\))-one (1.24) with 5-deoxy-\(\beta\)-arabinose gave a mixture of biopterin (1.23) and isobiopterin (1.25).\(^{40,42}\)

The ratio of the isomers is practically independent of the reaction conditions\(^{43}\) and almost unaffected by addition of hydrazine,\(^{44}\) boric acid,\(^{40}\) or \(p\)-toluidine,\(^{45}\) which all induce formation of the 6-isomers from hexoses - but do not effect the corresponding reaction with pentoses in a similar way.\(^{46}\) The 6- and 7-isomer of 1',2'-dihydroxypropylpterin can be separated by partition chromatography on silica\(^{47}\) or cellulose phosphate.\(^{48}\)

Biopterin was shown to have important biological function (see Section 1.3, p.27). However, the true biological activities of the other simple naturally occurring pterins are not yet known. A derivative of tetrahydrobiopterin occurs as an extremely photosensitive compound in the eyes and skin of insects, and in the skin of amphibia and fish.\(^49\) An \(\alpha\)-glucoside of biopterin has been isolated from the blue-green alga \textit{Anacystis nidulans}.\(^50\)

\textit{Ichthyopterin} was first isolated in 1943 from carp, and resembles biopterin structurally in being the '7-hydroxybiopterin'. Its structure was determined by synthesis from 6-acetonylisoxanthopterin.\(^{51}\) However, until the hypothetical \(S\)-erythro configuration of the
side-chain is established it should be called 6-(1,2-dihydroxypropyl)isoxanthopterin.

Rembold and Buschmann\textsuperscript{52} extracted pupae of some 2,000 bees with 3\% trichloroacetic acid, and were able to isolate and identify the following products:

<table>
<thead>
<tr>
<th>Product</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biopterin</td>
<td>0.4 mg</td>
</tr>
<tr>
<td>Pterin</td>
<td>0.04 mg</td>
</tr>
<tr>
<td>Pterin-6-COOH</td>
<td>0.025 mg</td>
</tr>
<tr>
<td>Isoxanthopterin</td>
<td>0.018 mg</td>
</tr>
<tr>
<td>Neopterin</td>
<td>0.03 mg</td>
</tr>
<tr>
<td>Violapterin</td>
<td>0.5 mg</td>
</tr>
</tbody>
</table>

Violapterin was shown to be 2,4,7-trihydroxypteridine.\textsuperscript{52}

Neopterin is 6-(1,2,3-trihydroxypropyl)pterin. Its absolute configuration was the subject of much discussion until Rembold and Buschmann\textsuperscript{53} proved it to be \textit{R-erythro} by unambiguous synthesis. According to Brown\textsuperscript{54} and Shiota\textsuperscript{55} \textit{R-erythro}-neopterin (cf. 1.26) is the common precursor formed in \textit{E.coli}.\textsuperscript{56} \textit{S-threo}-Neopterin is present in various organisms and is the main pterin found in \textit{E.coli} itself.\textsuperscript{57} Brown\textsuperscript{58} has also detected an enzyme which epimerized neopterin. His preliminary results indicated an alteration of the optical configuration of the intact dihydroneopterin side-chain:

\( (1.26) \rightarrow (1.27) \).

\textit{6-R-erythro}-Neopterin is proposed to participate in the pathway for the biosynthesis of folic acid\textsuperscript{59} and a number of other pterins.\textsuperscript{60} Numerous studies indicated that purines are utilized for pteridine ring
Scheme 1.2

Biosynthetic route to pterins

\[ R\text{-}erythro-7,8\text{-dihydroneopterin} \quad (1.26) \]

\[ R\text{-}threo-7,8\text{-dihydroneopterin} \quad (1.27) \]
formation in bacteria, fungi, amphibia and insects. Brown and coworkers have isolated the enzyme GTP-cyclohydrolase from *E. coli*. This enzyme catalyzes the conversion of guanosine-5'-triphosphate into \( R \)-erythro-7,8-dihydronopterin-3'-triphosphate (see Scheme 1.2). Recent data suggest alternate pathways for pteridine biosynthesis, e.g. addition of a two carbon unit to a derivative of 6-methyl-7,8-dihydropterin, and addition of a three carbon unit to 7,8-dihydropterin. Sepia and deoxysepiapterin (1.28 and 1.29 respectively) are often referred to as the yellow *Drosophila* pterins because they occur in relatively large amounts in the eyes of the *sepia* mutant of *Drosophila melanogaster*. The structures were revealed to be 6-substituted 7,8-dihydropterins with a lactyl side-chain for sepiapterin (1.28) and a propionyl substituent in the case of deoxysepiapterin (1.29). Separation of the two compounds was accomplished by chromatography. Deoxysepiapterin was synthesized in 1962 by Nawa and Forrest from 7,8-dihydropterin (1.30) and \( \alpha \)-ketobutyric acid in the presence of thiamine. Pfleiderer synthesized sepiapterin in a 20% yield, and determined its absolute configuration. The synthesis was by autoxidation of 5,6,7,8-tetrahydrobiopterin in buffer solution of pH 4. The separation of sepiapterin from the main reaction product biopterin (1.23) and small

\[ \text{† Deoxysepiapterin is commonly called "isosepiapterin". This is unfortunate because it is not isomeric with sepiapterin.} \]
amounts of pterin, pterin-6-carboxylic acid, and deoxysepiapterin was equally well evidenced by chromatography on a cellulose column. During the interconversion of tetrahydrobiopterin to pterin, Sepiapterin was good evidence for the configurational identity of the asymmetric atom C(2). Following the Fischer projection, Sepiapterin (1.28) belongs to the wedge series and should be called 6-(4-0-acetyl-7,8-dihydroxy-4H-pterin) acetate. Following the rules of Cahn, Ingold, and Prelog [68], the absolute configuration was determined by optical rotatory measurements. All naturally occurring pteridines discovered prior to 1966 were racemic. However, new aspects of pteridine chemistry were revealed through the work of Nasuda [69]. He reported the isolation of two new pteridine derivatives from the mycelium of *Fusarium oxysporum* [70]. Three years later, Neley and Plant [71] found two new substances in the mycelium of *Aspergillus terreus* [72, 73].

Structural studies and synthesis of these compounds (1.31) and (1.32) were not derivatives of pterin but of thiamine. Originally Nasuda called these two ribityl derivatives "G-substances" (1.31) and "M-substances" (1.32). The prefixes "G" and "M" represented the violet and green fluorescence of these compounds, respectively. After the structures became known, the names 6,7-dimethylthiamine for (1.31) and 6-methyl-7-hydroxymethylthiamine for (1.32) were proposed [74], but it is more correct to call the compounds ribityltimazines.
amounts of pterin, pterin-6-carboxylic acid, and deoxysepiapterin was readily achieved by chromatography on a cellulose column with water. The interconversion of tetrahydrobiopterin into sepiapterin was good evidence for the configurational identity of the asymmetric atom C(2').

Following the Fischer nomenclature, sepiapterin (1.28a) belongs to the L-series, and should be called 6-(S)-lactoyl-7,8-dihydropterin (1.28b) following the rules of Cahn, Ingold, and Prelog. The absolute configuration was confirmed by o.r.d. measurements.

All naturally occurring pteridines discovered prior to 1956 were derivatives of pterin. In 1956, however, new aspects of pteridine chemistry were revealed through the work of Masuda. He reported the isolation of two new pteridine derivatives from the mycelia of Eremothecium ashbyii. Three years later, Maley and Plaut found the same two substances in the mycelium of Ashbya gossypii yeast. Structural studies and syntheses showed that the two compounds (1.31) and (1.32) were not derivatives of pterin but of lumazine.

Originally Masuda called the two ribityl derivatives "G-substance" (1.31) and "V-substance" (1.32); the prefixes 'G' and 'V' referred to the green and the violet fluorescence respectively of these compounds. After the structures became known, the names 6,7-dimethylribolumazine for (1.31) and 6-methyl-7-hydroxyribolumazine for (1.32) were proposed, but it is more correct to call the compounds ribityllumazines.
After Masuda\textsuperscript{79} noted that the ultraviolet spectrum of "G-substance" resembled that of riboflavin (1.33), numerous studies of the enzymatic transformation of the former into riboflavin were undertaken.\textsuperscript{79-77} The results led to the hypothesis that 6,7-dimethyl-8-ribityllumazine (1.31) is an intermediate in the biosynthesis of riboflavin. Two molecules of 1.31 are joined by the catalytic action of the enzyme riboflavin synthetase to form a stable compound of riboflavin. This unusual biosynthetic reaction compound 1.31 is both a donor and acceptor for a 6,7-intermediate which consists of both the methyl groups and C(6) and C(7). A number of 6,7-substituted ribopyrimidines and 8-ribityllumazines have been synthesized to probe the substrate specificity of riboflavin synthetase. Some of these compounds are transformed into riboflavin in vitro. In contrast with 6,7-6-deoxyriboflavin) 6,7-dimethyl-8-ribityllumazine, which is a substrate for riboflavin through a chemical synthesis, they heated the 6,7-dimethyl-8-ribityllumazine for hours in a phosphate buffer and obtained riboflavin. Riboflavin (1.33) and 6-hydroxy-6-methyl-ribityllumazine are examples of 6,4-dimethyl-8-ribityllumazine found in nature.
After Masuda\textsuperscript{72} noted that the ultraviolet spectrum of "G-substance" resembled that of riboflavin (1.33), numerous studies of the enzymatic transformation of the former into riboflavin were undertaken.\textsuperscript{75} These led to the hypothesis\textsuperscript{71,75-77} that 6,7-dimethyl-8-ribityllumazine (1.31) was an intermediate in the biosynthesis of riboflavin, and not 7-hydroxy-6-methyl-8-ribityl-lumazine (1.32). Two molecules of 1.31 are joined by the catalytic action of the enzyme riboflavin synthetase to form one molecule of riboflavin. In this unusual biochemical reaction compound 1.31 is both a donor and acceptor for a C\textsubscript{4} intermediate which consists of both the methyl groups and C(6) and C(7). A number of 8-(R-1'-ribityl)-6,7-dialkylllumazines and 8-glycityl-6,7-dimethyl-lumazine had been synthesized in order to test the substrate specificity of riboflavin synthetase.\textsuperscript{78} None of these compounds were transformed into an isoalloxazine in contrast with 8-(R-5'-deoxyribityl)-6,7-dimethyl-lumazine which gave the respective isoalloxazine.\textsuperscript{77,78} All of these are, however, competitive inhibitors.

In 1963 Rowan and Wood\textsuperscript{79} succeeded in obtaining a 55\% yield of riboflavin through a chemical synthesis. They heated the lumazine (1.31) for fifteen hours in a phosphate buffer (pH 7.3) under nitrogen and obtained riboflavin.

7-Hydroxy\textsuperscript{80} and 6-hydroxymethyl-lumazine\textsuperscript{81} are examples of other 2,4-dioxopteridines found in nature.
1.1.4 The early history of folic acid and its derivatives *

During the 1930's a British medical practitioner, Lucy Wills, was concerned with a form of anaemia which occurred particularly in India and which was often associated with pregnancy. It was found that 'Marmite', a form of yeast extract, or crude liver extracts were effective in the treatment and prevention of this particular form of anaemia. Probably a new vitamin had been found. In the 1940's, several substances, which suppressed anaemia in mammals, had been isolated not only from liver and yeast but also from a number of other sources such as green leaves (folia). These "folic acids", as they were called, exhibited growth factor activity in some microorganisms. In 1946 an exciting discovery was made by a group of sixteen chemists from the Lederle Laboratories Division of the American Cyanamid Company, who reported the structure and synthesis of folic acid. It was pteroylglutamic acid (1.34), and the molecule was shown to be made up of a 6-methylpterin derivative condensed with p-aminobenzoic acid and glutamic acid.

1.1.5 Nomenclature and abbreviations of folic acid and its derivatives

The name "folic acid" was first proposed by Mitchell, Snell, and Williams in 1940 for the substrate which they isolated from spinach. It is now used specifically for pteroylglutamic acid. The numbering system most commonly used is shown in formula 1.34.

* For further reading see reference 82.
The term 'pteroylglutamic acid' is cumbersome when used in combination with a number of prefixes indicating substituent groupings - and the name is unfortunate because it suggests that the parent substance 'pteroid acid' is of more fundamental interest and importance than folic acid.

7,8-Dihydrofolic acid and 5,6,7,8-tetrahydrofolic acid are often abbreviated to DHF and THF respectively.

1.1.6 Further history of folic acid and its derivatives

Considerable confusion exists in the literature on the natural occurrence of folate derivatives prior to 1960. This is due partly to the multiplicity of these derivatives in most biological materials, and partly to the complexity of the chemical structures of these compounds. Estimation and identification were not too
reliable because of lack of specificity of the microbiological assay procedures. Paper chromatography was not adequate for the separation of natural mixtures of folate derivatives because many compounds are polybasic acids and highly polar, thus resembling each other very closely in chemical properties. Many derivatives are labile, reduced compounds, and are sensitive towards oxidation, hydrolysis, and photolysis. Already through research done on the first four natural folates (described in the following) general differences became apparent. Later research verified that naturally occurring folate derivatives differ from each other with regard to (a) the state of oxidation of the pteridine nucleus, (b) the nature of the one-carbon substituent at N(5), (c) the number of glutamate residues present in the molecule.

Folic acid itself (1.34) was isolated from yeast, autolyzed spinach, autolyzed hog liver, and aqueous extracts of liver. Its structure was determined by degradation studies and confirmed by synthesis around 1948 in the Lederle Laboratories. Equal molecular amounts of 2,4,5-triaminopyrimidin-6(1H)-one and p-aminobenzoylglutamic acid were dissolved in water and treated with 2,3-dibromopropionaldehyde. The yields were 30-50% of crude material which was shown to contain 10-25% of folic acid by microbiological assay.

Around the same time, folyldiglutamic acid (1.35) was isolated from an unidentified organism belonging to the genus Corynebacterium and its structure determined
Folyldiglutamic acid (n=1; 1.35)

Folylhexaglutamic acid (n=5; 1.36)
through its degradation products and confirmed by synthesis.

Folylhexaglutamic acid (1.36) was also isolated from yeast, and shown to be a polypeptide of folic acid.

Another folic acid derivative found a few years later in yeast was 5-formylTHF. Its structure was also determined by degradation and verified when a formyl group was introduced at N(5) in THF with a mixture of acetic anhydride and formic acid.

1.2 Stereochemical aspects of biopterin and folic acid

6-(1,2-Dihydroxypropyl)pterin can exist in four optical isomers:

As can be seen from formula (1.34), folic acid has an asymmetric centre in its side chain. When it is reduced to 5,6,7,8-THF (1.37), a second chiral centre is produced. Catalytic reduction with PDC in acetic acid gives an equal mixture of d-lactopine and L-pterin. The ratio of optical isomers is dependent on the specific enzyme and the stereochemistry of the THF used. The THF synthesized THF has the L-configuration at C(6), that is the 6,9-configuration.
Their characteristics are as follows:\(^1\)

<table>
<thead>
<tr>
<th></th>
<th>(R_f)</th>
<th>Biol. activ. (ng/ml)</th>
<th>([\alpha]^{25}_{589})</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-erythro</td>
<td>0.34</td>
<td>0.7</td>
<td>+61</td>
</tr>
<tr>
<td>S-erythro</td>
<td>0.34</td>
<td>0.006</td>
<td>-62</td>
</tr>
<tr>
<td>R-threo</td>
<td>0.16</td>
<td>0.5</td>
<td>-94</td>
</tr>
<tr>
<td>S-threo</td>
<td>0.16</td>
<td>0.2</td>
<td>+95</td>
</tr>
</tbody>
</table>

\(^*\) \(R_f\) values measured in isopropanol/5% boric acid soln. (4:1).

\(^**\) Crithidia half optimal growth concentration.

In attempts to separate the four isomers by chromatography only the erythro-form could be separated from the threo-form. For the further identification of all four isomers, the cofactor activity in the enzymatic reaction should be tested. The absolute configuration of the asymmetric centres in the side-chain of biopterin were deduced by synthesis from known sugars but the absolute configuration at C(6) of the tetrahydro derivative is not yet known.

As can be seen from formula (1.34), folic acid has an asymmetric centre in the \(S\)-glutamic acid side-chain. When it is reduced to 5,6,7,8-THF (1.37), a second chiral centre [at C(6)] is introduced. Catalytic reduction with \(\text{PtO}_2/\text{H}_2\) in acetic acid gives an equal mixture of diastereomers \([\alpha]^{27}_{589} +14.9^0\), \(\ell,S\) and \(d,S\) [i.e. \(\ell\) and \(d\) at C(6)]. Only one of these diastereoisomers is biologically active and the biologically synthesized THF has \([\alpha]^{29}_{589} -16.9^0\); that is the \(\ell,S\) form.\(^97\)
1.3 Biochemistry of mono-oxygenases that require reduced pterin cofactors

1.3.1 Pteridines in the hydroxylation of phenylalanine and tyrosine

The aromatic amino acid, \( \text{L-phenylalanine (1.38)} \), in higher organisms is converted to \( \text{L-tyrosine (1.39)} \), and initiates a catabolic as well as an anabolic pathway. The former is believed to be essential for the breakdown of phenylalanine to \( \text{CO}_2 \) and water. The anabolic part is the formation of \( \text{L-tyrosine with further conversion to the aromatic hormones, norepinephrine (1.42, noradrenaline) and epinephrine (1.43, adrenaline) via S-3,4-dihydroxyphenylalanine (1.40, dopa) and 2-(3,4-dihydroxyphenyl)ethylamine (1.41, dopamine) (see Scheme 1.3).}

Due to the above conversion, tyrosine is not an essential amino acid. In 1947 Jervis\(^{101}\) suggested that a block in the hydroxylation of phenylalanine is the reason for the disease phenylketonuria (phenylpyruvic oligophrenia, Folling's disease). It manifests itself in elevated levels of serum phenylalanine, urinary excretion of phenylpyruvic acid, and accumulation of phenylalanine metabolites leading to brain damage with severe mental retardation. Other symptoms are seizures and neurological abnormalities such as retarded myelination, and deficient melanin formation that predisposes eczema. This metabolic lesion is inherited and is autosomal recessive. It is relatively rare (occurs in about one out of every 25,000 births), but because a phenylalanine deficient diet can in many cases restore and reverse the condition, research in this area is of considerable medical importance.
Scheme 1.3

In 1953, results were published of both in vivo\textsuperscript{102} and in vitro\textsuperscript{103} studies which showed that the phenylalanine-tyrosine conversion is decreased in varying degrees in phenylketonuria patients.

The two proteins involved — a labile one from rat liver extracts and a stable one from sheep liver extracts — had similar activity in vitro and purified on a non-protein carrier. However, the complete structure of the cofactor had been elucidated. Chemical evidence suggested that pteridine might be the active component.\textsuperscript{109} The first compound of this group to show activity was tetrahydrofolic acid (THF).\textsuperscript{109} The tetrahydro derivatives of 6-methyl- (1.46) and 6,7-dimethylpterin (1.47) were also found to be active and gave more effective than THF (see Figure 1.1).\textsuperscript{110}
In 1953, results were published of both *in vitro*\(^{102}\) and *in vivo*\(^{103}\) studies which showed that the phenylalanine-tyrosine conversion is decreased in varying degrees in phenylketonuric patients.

In 1956-57 it was shown\(^{104-106}\) that the system which catalyzes the hydroxylation of phenylalanine requires at least two protein fractions. In phenylketonuria the levels in various tissues are low in one of the fractions (phenylalanine hydroxylase), and in lethal hyperphenylalaninemia the levels of the second protein fraction (dihydropterin reductase) are low.\(^{107}\)

After having studied the stoichiometry of the enzymatic conversion [Scheme 1.3; \((1.38) \rightarrow (1.39)\)], Kaufman\(^{106}\) formulated the following reaction:

\[
\text{NADPH} + H^+ + O_2 + \text{phenylalanine} \rightarrow \text{NADP}^+ + H_2O + \text{tyrosine}.
\]

The two proteins involved - a labile one from rat-liver extracts and a stable one from sheep-liver extracts - had been partially purified. In 1958 Kaufman\(^{108}\) further showed that in addition to the two enzyme fractions, this enzyme system requires a non-protein cofactor which was purified extensively from rat liver. Long before the complete structure of the cofactor had been elucidated, chemical evidence suggested that a pteridine might be the active component.\(^{109}\) The first compound of this group to show activity was tetrahydrofolic acid (THF).\(^{109}\) The tetrahydro derivatives of 6-methyl- (1.46) and 6,7-dimethyl-pterin (1.47) were also found to be active and were more effective than THF (see Figure 1.1).\(^{110}\)
Figure 1.1
(from Ref. 110)
The isomeric 4-amino-2-hydroxy-6-methyl-5,6,7,8-tetrahydropteridine, and folic acid, dihydrofolic acid, 5-formyltetrahydrofolic acid and non-reduced pteridine were all inactive.

Kaufman arrived at the conclusion that the primary site of oxidation of the tetrahydropterin is N(5), and that the primary oxidation product has a 'quinonoid' structure, by considering the relative rates of aerial oxidation of the tetrahydropterins and the relative rates of reduction of the corresponding dihydropterins by NADPH. Much evidence has since been presented in support of the intermediate 'quinonoid' 6,7-dihydropterin (1.44) which could not be isolated because it rearranged rapidly in the absence of enzymes to the isomeric biologically inactive 7,8-dihydropterin (1.45).

The enzyme purified from rat-liver is called phenylalanine hydroxylase and the NADPH requiring enzyme purified from sheep-liver is named quinonoid-dihydropterin reductase (or, less specifically, dihydropteridine reductase). The latter reduces the 'quinonoid' dihydropterin formed back to its tetrahydro derivative; and the cycle (see Scheme 1.4) is then repeated.

The first two steps (1.38 + 1.39 + 1.40) in the biosynthetic pathway depicted in Scheme 1.3, involve this cycle. A similar cycle is found in the conversion of S-tryptophane (1.48) to S-5-hydroxytryptophane (1.49).
Scheme 1.4

\[ \text{NADPH} \rightarrow \text{Dihydropteridine Reductase} \]

\[ \text{O}_{\text{2}} \rightarrow \text{Hydroxylase} \]

\[
\begin{align*}
\text{5-Hydroxytryptophan} & \quad \text{(1.48)} \\
\text{S-Tryptophan} & \quad \text{(1.49)}
\end{align*}
\]

\[
\begin{align*}
(1.50a) & \quad R = H \\
(1.50b) & \quad R = \text{CH}_3
\end{align*}
\]
Oxidized pterins and pteridines are often the end products obtained from the isolation of naturally occurring compounds. It is, however, the di- or tetrahydro derivatives that play the functional role as cofactors in a number of oxygenase reactions.

In 1962, Kaufman\textsuperscript{113} found that extracts from the \textit{sepi}a mutant of \textit{Drosophila melanogaster} had high cofactor activity. An active compound was isolated and identified as sepiapterin (1.28, p.16). It was the first naturally occurring, simple pteridine to show activity. In the same paper, Kaufman reported that biopterin (1.23, p.9), another pteridine found in the \textit{sepi}a mutant, also showed high activity but only after chemical reduction. After the u.v. absorption spectra and other characteristics of reduced biopterin were known, it was found that the cofactor purified from rat-liver was indeed dihydrobiopterin. Studies of its growth-promoting properties in the trypanosomid flagellate \textit{Crithidia fasciculata} established that the 1,2-dihydroxypropyl side-chain probably has the \textit{S}-\textit{erythro} configuration which was confirmed after the \textit{S}-\textit{erythro} isomer was synthesized unambiguously by several independent workers\textsuperscript{41,114,115} from 5-deoxy-\textit{S}-arabinose.

Tyrosine hydroxylase, a related oxygenase, has been highly purified from bovine adrenal medulla.\textsuperscript{116} Tetrahydrobiopterin also seems to be the natural cofactor for this enzyme.\textsuperscript{117}
1.3.3 Folate cofactors

Blakley,\textsuperscript{118} and Benkovic and Bullard\textsuperscript{119} have summarized the role of 5,6,7,8-tetrahydrofolic acid in the metabolism of single carbon units at the various levels of oxidation. The major pathways and reactions involving these cofactors have been well described. The amount of unambiguous and instructive physical and chemical data is, however, limited. The identity of certain enzymes requiring folate cofactors is still in question due to contaminating and overlapping activities. The mechanisms of enzymatic catalysis of many reactions involving THF derivatives are therefore not yet completely understood.

It is known that exogenous folic acid, and 7,8-dihydrofolic acid formed in the biosynthetic pathway, both require dihydrofolate reductase to catalyze their reduction to the tetrahydro level. Blakley\textsuperscript{120} has outlined the properties of the enzyme isolated from various sources. THF is an essential coenzyme for the biosynthesis of inosinic acid, the biological precursor in the \textit{de novo} synthesis of purine nucleotides. Also, \textit{N}(5),\textit{N}(10)-methylene-THF is the coenzyme for thymidine synthetase which converts deoxyuridine monophosphate to thymidylic acid, and is necessary for the synthesis of deoxyribonucleic acids (DNA). Inhibition of the formation of THF will affect nucleic acid biosynthesis, which ultimately leads to the death of the cell.\textsuperscript{119,121}

Much work has been done in attempts to synthesize folic acid derivatives that specifically and irreversibly inhibit the action of dihydrofolate reductase.\textsuperscript{122,123}
Scheme 1.5

INTERCONVERSIONS OF THF DERIVATIVES IN RELATION TO THE GENERATION AND UTILIZATION OF ONE-CARBON UNITS

(based on Figure 1.6, p.188 in ref. 23)
The most potent are aminopterin (1.50a) and amethopterin (Methotrexate, 1.50b) which inhibit the enzyme owing to a high binding constant (>10⁹).

As mentioned earlier, it is in general as tetrahydro derivatives that folate and other pterins participate in metabolism. However, THF is not the coenzyme in all the enzymatic reactions requiring folate cofactors because its 5-formyl, 5-methyl and other derivatives from THF also are cofactors for specific enzymatic reactions. The enzymatic conversions of THF to these derivatives are summarized in Scheme 1.5. Reactions leading to the formation of compounds containing an 'active' one-carbon unit are depicted in the upper part of the Scheme. The biosynthetic function of these 'activated' units is outlined in the lower part of the Scheme. The equations 1.37 ⇄ 1.51 ⇄ 1.52 ⇄ 1.53 show the interconversion of THF and its various derivatives.

1.4 Scope of problems

The absolute configuration at C(6) is not yet known in 5,6,7,8-tetrahydrofolic acid and in the reduced pterins which are active as cofactors in the hydroxylation of phenylalanine (Scheme 1.3 and 1.4), such as 6-methyl- and 6,7-dimethyl-5,6,7,8-tetrahydropterin, and the natural cofactor 5,6,7,8-tetrahydrobiopterin. The available data indicate that the chiral centre at C(6) and the chiral or prochiral centre at C(7) in the latter pterins [(1.46), (1.47), and (1.54)] are unaffected in the enzymatic cycle (Scheme 1.4). More direct evidence regarding the integrity of the relative configuration of these two
centres during the cycle could provide a better understanding of the mechanism involving the two enzymes (i.e., does a double inversion take place?). Knowledge is also lacking about the relative and absolute stereospecificities at these two centres when the substrate, e.g., 6-methylpterin or folic acid, is reduced by folate reductases.

A spectroscopic method to identify the stereochemistry of addition of hydrogen to both 6-methyl- and 5,6,7,8-tetrahydropterins was recommended in order to solve some of these questions. It is suggested that the synthesis of the two substrates and their deuterated derivatives to quantify the interpretation of recorded spectra is needed.

In a study of the relative effectiveness of pterin cofactors and tetrahydrofolic acid in the hydroxylation of phenylalanine to tyrosine system (Scheme 3.4), Kaufman and Kaufman found that synthetic 6-tetrahydrofolic acid (racemic at C(6)) was more effective than the biologically active isomer 4-tetrahydrofolic acid. The study indicated that there may be some stereospecific interaction between the enzyme and the cofactors. The enantiomers of 6-methyl-5,6,7,8-tetrahydropterin should be very useful for examining their relative efficiency in comparison with the racemate towards both enzymes: phenylalanine hydroxylase and dihydropterin reductase separately and together in the mono-oxygenase system. An optical resolution of 6-methyl-5,6,7,8-tetrahydropterin is therefore warranted.

5,6,7,8-TETRAHYDROBIOPTERIN

(1.54)
centres during the cycle could provide a better understanding of the mechanism involving the two enzymes (i.e. does a double inversion take place?). Knowledge is also lacking about the relative and absolute stereospecificities at these two centres when the substrate, e.g. 6-methylpterin or folic acid, is reduced by folate reductases.

A spectroscopic method to identify the stereochemistry of addition of hydrogen to C(6) and C(7) in 6-methyl- and 6,7-dimethyl-pterin was required in order to solve some of these problems. This suggested the synthesis of the two substrates and their deuterated derivatives to simplify interpretation of the recorded spectra in a p.m.r. approach.

In a study of the relative effectiveness of pterin cofactors and tetrahydrofolic acid in the hydroxylation of phenylalanine to tyrosine by the mono-oxygenase system (Scheme 1.4), Kaufman\(^1\) found that synthetic $\text{dl}$-tetrahydrofolic acid [racemic at C(6)] was more effective than the biologically active isomer $\epsilon$-tetrahydrofolic acid. The study indicated that there may be some stereospecific interaction between the enzyme and the cofactors. The enantiomers of 6-methyl-5,6,7,8-tetrahydropterin should be very useful for examining their relative efficiency in comparison with the racemate towards both enzymes phenylalanine hydroxylase and dihydropterin reductase separately and together in the mono-oxygenase system. An optical resolution of 6-methyl-5,6,7,8-tetrahydropterin is therefore warranted.
The determination of the absolute configuration of 6-methyl-5,6,7,8-tetrahydropterin is necessary not only for a further understanding of the mechanism of the above enzymes but also for other studies in Dr Armarego's laboratory towards establishing the absolute configuration at C(6) of the biologically active L-tetrahydrofolic acid. Means of stabilizing 6-methyl-5,6,7,8-tetrahydrofolic acid have to be found in order to degrade the optical isomers without affecting the asymmetric centre at C(6). It is thus necessary to examine the stability of 5,6,7,8-tetrahydropterins and derivatives which could be used for degradation studies.

Experiments directed towards the solution of some of these problems are described in this thesis.
STEREOCHEMISTRY OF CATALYTIC REDUCTION OF 6-METHYL- AND
6,7-DIMETHYL-PTERINS AND OF 2,4-DIAMINO-6-METHYLPTERIDINES

This Part includes an account of the preparation and reduction of various deuterated derivatives (2.2 - 2.4, 2.14 and 2.6) of 6-methylpterin (2.1), 2,4-diamino-6-methylpteridine (2.13) and 6,7-dimethylpterin (2.5). The relative stereochemistry of catalytic hydrogen addition to C(6) and C(7) in these compounds is deduced from the p.m.r. spectra of the tetrahydro derivatives (2.7 - 2.12, 2.15 and 2.16).

2.1 General syntheses of pteridines

Prior to 1973, a high proportion of all known synthetic pteridines were prepared by some modification of the classical Isay method involving the condensation of a 4,5-diaminopyrimidine with an α,β-dicarbonyl compound.11,26 The reason for the usefulness of this method is that it is flexible due to the numerous possibilities for substitution in the starting materials which generally condense giving high yields. The method is, however, not suitable for preparation of 6-substituted pteridines exclusively because condensation involving α-ketoaldehydes give predominantly the 7-substituted isomer contaminated with the 6-isomer. This is so because the relatively more active carbonyl, the aldehyde group, reacts more readily with the more nucleophilic 5-amino group (see Scheme 2.1).26 The separation of the two isomers may be difficult or even impossible.
<table>
<thead>
<tr>
<th>Equation</th>
<th>R&lt;sup&gt;1&lt;/sup&gt;</th>
<th>R&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>(2.1)</td>
<td>R&lt;sup&gt;1&lt;/sup&gt;=R&lt;sup&gt;2&lt;/sup&gt;=H</td>
<td></td>
</tr>
<tr>
<td>(2.2)</td>
<td>R&lt;sup&gt;1&lt;/sup&gt;=H, R&lt;sup&gt;2&lt;/sup&gt;=D</td>
<td></td>
</tr>
<tr>
<td>(2.3)</td>
<td>R&lt;sup&gt;1&lt;/sup&gt;=D, R&lt;sup&gt;2&lt;/sup&gt;=H</td>
<td></td>
</tr>
<tr>
<td>(2.4)</td>
<td>R&lt;sup&gt;1&lt;/sup&gt;=R&lt;sup&gt;2&lt;/sup&gt;=D</td>
<td></td>
</tr>
<tr>
<td>(2.5)</td>
<td>R&lt;sup&gt;1&lt;/sup&gt;=H, R&lt;sup&gt;2&lt;/sup&gt;=CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>(2.6)</td>
<td>R&lt;sup&gt;1&lt;/sup&gt;=D, R&lt;sup&gt;2&lt;/sup&gt;=CD&lt;sub&gt;3&lt;/sub&gt;</td>
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<tr>
<td>(2.7)</td>
<td>R&lt;sup&gt;1&lt;/sup&gt;=R&lt;sup&gt;2&lt;/sup&gt;=H</td>
<td></td>
</tr>
<tr>
<td>(2.8)</td>
<td>R&lt;sup&gt;1&lt;/sup&gt;=H, R&lt;sup&gt;2&lt;/sup&gt;=D</td>
<td></td>
</tr>
<tr>
<td>(2.9)</td>
<td>R&lt;sup&gt;1&lt;/sup&gt;=D, R&lt;sup&gt;2&lt;/sup&gt;=H</td>
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<tr>
<td>(2.10)</td>
<td>R&lt;sup&gt;1&lt;/sup&gt;=R&lt;sup&gt;2&lt;/sup&gt;=D</td>
<td></td>
</tr>
<tr>
<td>(2.11)</td>
<td>R&lt;sup&gt;1&lt;/sup&gt;=H, R&lt;sup&gt;2&lt;/sup&gt;=CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>(2.12)</td>
<td>R&lt;sup&gt;1&lt;/sup&gt;=D, R&lt;sup&gt;2&lt;/sup&gt;=CD&lt;sub&gt;3&lt;/sub&gt;</td>
<td></td>
</tr>
</tbody>
</table>

![Chemical structures](image)
Isay's Pteridine Synthesis

Scheme 2.1
Much effort has been devoted to devise reaction conditions which would cause reversal of the normal direction of condensation. Control of pH, addition of hydrazine, sodium bisulphite or 2-mercaptoethanol, and the use of $\alpha$-hydroxy-ketones rather than $\alpha,\beta$-dicarbonyl compounds had generally been partially successful. 26

Other unequivocal condensations have been tried, such as (a) the reaction of a 4-amino-5-nitrosopyrimidine with an active nitrile-substituted methylene compound (the Timmis reaction), 125 (b) the Boon and Leigh synthesis involving condensation of a 4-chloro-5-nitropyrimidine with an $\alpha$-amino-aldehyde or ketone, followed by reductive cyclization, 126 (c) the condensation of a 4-amino-5-nitrosopyrimidine with phenacyl- or acetonyl-pyrimidinium salts, 127 and (d) the use of pyrazine intermediates which finally required closure of the fused pyrimidine ring. 128

The last method, however, had the drawback that suitable substituted pyrazine intermediates (i.e. 2-aminopyrazine-3-carboxylic acid derivatives) were difficult to prepare.

In 1973, E.C. Taylor and coworkers 129 published the first paper in a series describing a general synthetic route to 6-substituted pteridines which is unequivocal in the orientation of the 6- or 7-substituent. 2,4-Diamino-6-substituted pteridines, for example, were made by treating $\alpha$-ketoaldoximes (e.g. 2.19) with aminomalono-nitrile (2.17) which gave 2-amino-3-cyano-5-substituted pyrazine 1-oxides (e.g. 2.22). These oxides were subsequently converted to 2,4-diamino-6-substituted pteridine 8-oxides (e.g. 2.38) by condensation with guanidine in the presence of methoxide. Many of the
(2.17) $R = \text{CN}$  
(2.18) $R = \text{COOEt}$  
(2.19) $R^1 = R^2 = H$  
(2.20) $R^1 = H, R^2 = D$  
(2.21) $R^1 = R^2 = D$  
(2.22) $R = \text{CN}, R^1 = R^2 = H$  
(2.23) $R = \text{CN}, R^1 = H, R^2 = D$  
(2.24) $R = \text{CN}, R^1 = R^2 = D$  
(2.25) $R = \text{CONH}_2, R^1 = R^2 = H$  
(2.26) $R = \text{COOEt}, R^1 = R^2 = H$  
(2.27) $R = \text{COOEt}, R^1 = H, R^2 = D$  
(2.28) $R = \text{COOEt}, R^1 = R^2 = D$  
(2.29) $R = \text{CN}, R^1 = R^2 = H$  
(2.30) $R = \text{CN}, R^1 = R^2 = D$  
(2.31) $R = \text{CONH}_2, R^1 = R^2 = H$  
(2.32) $R = \text{COOEt}, R^1 = R^2 = H$  
(2.33) $R = \text{COOEt}, R^1 = H, R^2 = D$  
(2.34) $R = \text{COOEt}, R^1 = R^2 = D$
pteridine O-oxides could be reduced to the corresponding 2,3-dihydropteridines (e.g. 2.35) by sodium dithionite. Subsequent oxidation with potassium permanganate (or even with glucose) gave 2,4-diamino-5-substituted pteridines (e.g. 2.36). Alternatively, deoxygenation of the pteridine 1-oxides (e.g. 2.22) with either phosphorus (III) or sodium dithionite gave pteridines (e.g. 2.29).

The flavin 3-oxo-3-carboxylic acid (e.g. 2.19) or its dihydro derivative (e.g. 2.30) were converted, with quinoline in basic solution, to an 8-substituted pteridine-6-oxo-3-carboxylic acid (e.g. 2.35). The analogous 5,6-dihydropteridines (e.g. 2.36) were oxidized by hypobromite, followed by acetic acid to give the corresponding 2,3-dihydropteridines (e.g. 2.36) and dehalogenation, respectively. These were used in the synthesis of 8-deuteriopteridines.

The synthesis of 8-deuteriopteridines, 6-deuterio-pteridines, and 7-deuterio-pteridines was achieved by modification of the method employed for the synthesis of 6-deuterio-pteridines (2.35) from 2,4,5-triaminopyrimidine-6(4H)-one and peracetic acid with addition of sodium sulphite and sodium carbonate. The contaminating 7-isomer (2.35) was removed by recrystallization from 28-sodium hydroxide.
pteridine 8-oxides could be reduced to the corresponding 7,8-dihydropteridines (e.g. 2.39) by sodium dithionite. Subsequent oxidation with potassium permanganate (or even air) gave 2,4-diamino-6-substituted pteridines (e.g. 2.13). Alternatively, deoxygenation of the pyrazine 1-oxides (e.g. 2.22) with either phosphorus trichloride or sodium dithionite gave pyrazines (e.g. 2.29) which condensed with guanidine to yield pteridines (e.g. 2.13).

6-Substituted pterins were synthesized similarly by treating an α-ketoaldoxime (e.g. 2.19) or an α-keto-ketoxime with esters of α-aminocyanacetic acid (e.g. 2.18). The resulting 3-alkoxycarbonyl-2-aminopyrazine 1-oxides (e.g. 2.26) were converted, with guanidine in basic solution, to 6-substituted pterin 8-oxides (e.g. 2.35). The latter (like the analogous aminopteridine 8-oxides) could be reduced by dithionite followed by a permanganate oxidation to give 7,8-dihydropterins (e.g. 2.36) and pterins (e.g. 2.1) respectively. This last synthesis has been very satisfactory for the present work, although some difficulties were encountered.

2.2 Synthesis of 6-methyl-, 6-trideuteromethyl-, 7-deutero-6-methyl- and 7-deutero-6-trideuteromethyl-pterins

6-Methylpterin (2.1) was prepared by a modified Isay-method from 2,4,5-triaminopyrimidin-6(1H)-one and pyruvaldehyde with addition of sodium sulphite and pyrosulphate. The contaminating 7-isomer (36%) was removed by recrystallization from 2N-sodium hydroxide.
The first approach to the preparation of deuterated 6-methylpterins was by direct deuteration of 6-methylpterin. Deuterated hydrochloric acid, however, caused very little exchange, other than of the labile hydrogen atoms on N(3) and the 2-amino group. Sodium deuteroxide in deuterium oxide, on the other hand, exchanged all the protons on the C(6)-methyl group, but there was only negligible exchange of H(7) under the variety of conditions tried. 6-Trideuteromethylpterin (2.3 of >98% isotopic purity) was successfully prepared in this way.

The main problem was now to find a way of incorporating a deuterium atom on C(7) in 6-methylpterin. The preparation of 7-deutero-6-trideuteromethylpterin would then readily follow from the above deuterium exchange.

It was possible to partially deuterate 6-methylpterin by reducing it with dithionite in deuterium oxide. This treatment would cause addition of deuterium atoms to both C(7) and N(8) and give 7,8-dideutero-6-methylpterin (2.37). However, theoretically half the deuterium on C(7) would be lost on reoxidation with permanganate. By repeating the procedure, the deuterium content was brought up to ~75% but in only 41% yield, so the method was generally unsatisfactory because high deuterium incorporation, which was necessary for subsequent work, could be obtained only in very low yields.

The preparation of deuterated pteridines was then attempted by using the unambiguous pteridine synthesis of Taylor and coworkers. The starting materials
for the synthesis of 2-amino-6-deutero-3-ethoxycarbonyl-5-methylpyrazine 1-oxide (2.27) are 1-deutero-1-hydroxyiminoacetone (2.20) and ethyl α-aminocyanacetate 3-p-toluenesulphonate (2.18). The latter salt was also needed, together with perdeuterated hydroxyiminoacetone (2.21) for the preparation of 2-amino-6-deutero-3-ethoxycarbonyl-5-trideuteromethylpyrazine 1-oxide (2.28).

Direct deuterium exchange reactions in hydroxyiminoacetone (2.19) turned out to be unsatisfactory. In deuterium oxide, the exchange was very slow, and on addition of sodium deuteroxide, the protons of the methyl group exchanged more rapidly than the aldehydic proton. P.m.r. spectra of aliquots showed that a second isomer was formed during the exchange reaction in deuterium oxide both with and without sodium deuteroxide. This was probably due to syn-anti isomerism. It is known that the formyl proton syn to the hydroxyl group in oximes resonates downfield from the corresponding anti proton ($\Delta\delta = 0.17$ p.p.m. in the present case). This means that the hydroxyiminoacetone consisted mainly of the anti isomer, and that the syn isomer was gradually formed during the D/H exchange. Although almost complete exchange of the aldehydic proton was possible after long treatment, it was found that during the exchange of the CD$_3$ group back to a CH$_3$ group with aqueous sodium hydroxide, in order to form the 1-deutero-1-hydroxyiminoacetone, much self condensation had taken place. This was revealed in the p.m.r. spectrum by an increase in the number of signals from methyl groups on saturated
carbons. Also, 2N-hydrochloric acid caused considerable self condensation.

1-Deutero-1-hydroxyiminoacetone (2.20), for the preparation of 6-deuteropyrazine 1-oxides (2.23 and 2.27), was made in an almost quantitative yield by treating t-butyl α-nitrosoacetoacetate (2.40, from nitrosation of t-butyl acetoacetate) with deuterotrifluoroacetic acid. The deuterated acid decomposed the ester, and decarboxylated the acid formed to insert a deuterium at C(1).

Perdeuterated hydroxyiminoacetone (2.21) of high deuterium content was conveniently prepared by modifying Vanino's method for the non-deuterated compound134 (see Scheme 2.2 and Experimental).

The second component for the synthesis of deuterated 2-amino-3-ethoxycarbonyl-5-methylpyrazine 1-oxides (2.27, 2.28) was ethyl α-aminocyanoacetate p-toluenesulphonate (2.18) which did not need to contain any deuterium atoms since these would later be lost in the reaction with guanidine. The salt of the amino compound (2.18) was prepared at the same time and in the same way as aminomalononitrile p-toluenesulphonate (2.17, to be used later for preparation of cyanopyrazines). The method chosen was that of Ferris, Sanchez and Mancuso135 (see Scheme 2.3). Both ethyl cyanoacetate and malononitrile were nitrosated completely as shown by p.m.r. spectroscopy, and (for malononitrile) by formation of the hydroxyiminomalononitrile silver salt from which the free nitroso derivative was liberated. However, many problems were encountered in the reduction of the nitroso compounds with aluminium amalgam (Equation b in Scheme 2.3) and in the precipitation
\[
\text{HO-} \text{N=CD} + (\text{CH}_3)_2\text{C=CH}_2 + \text{CO}_2
\]

(2.20)

\[
\text{HO-} \text{N=CD} + \text{COO}Bu^+ + \text{CF}_3\text{COOD}
\]

(2.40)

\[
\text{HO-} \text{N=CD} + (\text{CH}_3)_2\text{C=CH}_2 + \text{CO}_2
\]

(2.20)

\[
\text{CH}_3\text{COCH}_2\text{COOEt} + \text{NaOD} + \text{NaNO}_2
\]

\[
\text{CD}_3\text{COCD}_2\text{COO}^- + 2\text{Na}^+ + \text{OD}^- + \text{NO}_2^- + \text{EtOD}
\]

(2.21)

**Perdeuterated Hydroxyiminoacetone**

**Scheme 2.2**
Equation a: \[ \text{CH}_2\text{CN} + \text{NaNO}_2 + \text{CH}_3\text{COOH} \rightarrow \text{HO}-\text{N}=\text{C} + \text{CH}_3\text{COONa} + \text{H}_2\text{O} \]

Equation b: \[ 3 \text{HO-N}=\text{C} + 4 \text{Al(Hg)} + 9 \text{H}_2\text{O} \rightarrow 3 \text{H}_2\text{N-CH} + 4 \text{Al(OH)}_3 + \text{Hg} \]

Equation c: \[ \text{H}_2\text{N-CH} + \text{H}_3\text{C-SO}_3\text{H} \]

(2.17) \( R = \text{CN} \) Aminomalonenitrile \( p \)-toluenesulphonate

(2.18) \( R = \text{COOEt} \) Ethyl \( \alpha \)-aminocyanooacetate \( p \)-toluenesulphonate

Scheme 2.3
of the aminonitriles formed, as the p-toluenesulphonates. The difficulties encountered and improvements suggested for the preparations are described in the Experimental Section, p.99. The aminomalononitrile was found to be unstable and polymerized on standing at room temperature during approximately one hour unless it was converted into a salt.

The reaction of ethyl α-aminocyanocacetate p-toluene-sulphonate (2.18) with perdeuterated hydroxyiminoacetone (2.21) proceeded smoothly and gave 2-amino-6-deutero-3-ethoxy carbonyl-5-trideutero methylpyrazine 1-oxide (2.28) in which only a small proportion (~1/10) of the deuterium in the methyl group and none of the ζ(6)-deuterium had been exchanged. Complete deuteration of the pyrazine ester could be obtained by direct (but slow) deuteration exchange in 6N-deuterium oxide.

2-Amino-6-deutero-3-ethoxy carbonyl-5-trideutero methylpyrazine 1-oxide with high deuterium content (~100% D on C(6) and ~85% D on ζ(5)-C) reacted with guanidine in the presence of sodium methoxide, and although 6-methylpterin 8-oxide was obtained in high yield, almost complete exchange of deuteron at C(7) and in the methyl group had occurred in the strongly basic medium. If an excess of methoxide was not used, the condensation did not take place. A repetition of this reaction with deuterated solvents would be prohibitive in cost. The alternative was to attempt a condensation between the deoxygenated deutero pyrazine 1-oxide (2.34) and guanidine.

When 2-amino-6-deutero-3-ethoxy carbonyl-5-trideutero pyrazine 1-oxide was reduced catalytically (Pd/C) in
ethanol, p.m.r. spectroscopy showed that the amount of deuterium lost from C(6)(0-85%) and from CD₃(5)(0-20%) increased if the duration of the reduction was increased [15-90 min (50% and 5% Pd/C respectively)]. That hardly any deuterium was lost when the catalytic reduction was carried out in 15 min, using 50% Pd/C, suggested that the deoxygenation had taken place by oxygen abstraction from the nitrogen and not that hydrogen addition to the oxygen atom and C(6) had occurred with subsequent loss of water. In the latter event this would theoretically have removed half of the deuterium on C(6). The loss of deuterium on shaking for a longer period must therefore have been a result of direct D/H exchange under the influence of hydrogen and the catalyst. Shaking the ester with catalyst alone did not cause any hydrogen exchange.

The above considerations became less important when the attempt to condense ethoxycarbonylpyrazine (2.34) with guanidine revealed that deoxygenation had rendered the ester unreactive towards guanidine.

Attempts were then made to prepare 7-deuterated 6-methylpterin and its 8-oxide by condensing a reactive reagent, such as cyanamide and cyanogen bromide among other reagents, with pyrazines or their 1-oxides which could be obtained with a deuterium atom at C(6). However, 2-amino-3-ethoxycarbonyl-5-methylpyrazine 1-oxide (2.26) did not react with either cyanamide or S-methyl isothiouronium sulphate. 2-Amino-3-carbamoyl-5-methylpyrazine 1-oxide (2.25), formed by treating the nitrile (2.22) with sulphuric acid, did not condense with guanidine in the presence of methoxide, cyanamide, cyanogen bromide or
ammonium thiocyanate. Also the preparation of a pterin from 3-carbamoyl (2.31) or 3-ethoxycarbonyl (2.32) 2-amino-5-methylpyrazine and guanidine in the presence of methoxide was unsuccessful. These data imply that in the formation of the pteridine 8-oxide, guanidine reacts first with the nitrile or ester function in 3-cyano- or 3-ethoxycarbonyl-pyrazine 1-oxides respectively, and intramolecular cyclization then takes place onto the 2-amino group. If this is not the case, then the 2-amino-3-ethoxycarbonyl-5-methyl pyrazine 1-oxide should be less reactive than the corresponding deoxygenated pyrazine because of the stronger basicity of the amino group in the latter. 2-Amino-3-cyano-5-methylpyrazine 1-oxide (2.22) was prepared from aminomalnonitrile and hydroxyiminoacetone because it was predicted that the nitrile function would be more reactive than the ester function in this system. The pyrazine 1-oxide (2.22) was deoxygenated as described above, and the pyrazine formed (2.29) was condensed with guanidine which gave 2,4-diamino-6-methylpteridine (2.13) [see Section 2.3, p.57 for synthesis].

The reason for making the 2,4-diaminopteridine was that it should be possible to convert daminopteridines to the corresponding pterins by mild acid or base hydrolysis. Contrary to expectations, it was found that several 2,4-diaminopteridines were unaltered by nitrous acid regardless of the substitution on the pyrazine part of the pteridine nucleus. Taylor and
Cain showed, however, that although an amino group on C(4) is resistant to nitrous acid, it may be removed readily by hydrolysis with dilute mineral acid. They converted 2,4-diaminopteridine and 2,4-diamino-6,7-dimethylpteridine into the corresponding pterins in good yields by boiling with 6N-hydrochloric acid for a short time (30 min). Upon boiling for a longer period (30 h) even the amino group on C(2) could be hydrolysed. Later, Taylor and Jacobi reported that 2,4-diaminopteridine 8-oxide could be converted to pterin 8-oxide by alkaline hydrolysis in 98% yield, but they did not give experimental details. In connection with the present work, it was found that 2,4-diamino-6-methylpteridine could be converted to 6-methylpterin in boiling 48% hydrogen bromide during 30 min and in boiling concentrated hydrochloric acid during an hour in ~75% yields, whereas the corresponding diaminopteridine 8-oxide required boiling overnight in 2N-sodium hydroxide before the conversion was almost complete (90%). Nitrosation of the diaminopteridine 8-oxide failed to give pterin 8-oxide, while boiling with 1N-sodium hydroxide and 3N-hydrochloric acid were partially successful. The reason for the difference in ease of hydrolysis of amino groups on C(2) and C(4) could be (as proposed by Taylor and Cain) that NH₂(2) is part of a guanidine structure whereas NH₂(4) is part of an amidine structure. It is recognized that guanidine
is much more stable to hydrolysis in acid medium than is an amidine. It is also known that aniline is increasingly difficult to diazotize when the number of electron-attracting substituents is increased. A similar effect in pteridines could be the reason why nitrous acid failed to react with 2,4-diamino-6-methylpteridine 8-oxide. In the pyrimidine part of the ring, both N(1) and N(3) can gain a positive charge in acid solution and act as effective electron-withdrawing groups. These electron-attracting properties, which are further increased by the presence of the π-electron deficient pyrazine ring, would (if the above theory is correct) have the effect of rendering the pteridine less reactive towards nitrous acid.

It was then investigated to what extent hydrogen would be exchanged with deuterium if 2N-sodium deuterioxide was used in the above hydrolysis of 2,4-diamino-6-methylpteridine. As expected, the diamino compound was converted to a pterin which, by p.m.r. spectroscopy, was shown to be fully deuterated on the C(6)-methyl group (>98% of isotopic purity) whereas only negligible exchange of H(7) had occurred. This is in accordance with what is already known about D/H exchange in 6-methylpterin when treated with 2N-sodium deuterioxide (see p.47).

The study of the hydrolysis of diaminopteridines to the corresponding pterins resulted in a very
satisfactory way of preparing 6-methylpterins. This involved heating and recrystallizing the 2,4-diamino derivative from 2N-sodium hydroxide, which yielded the sodium salt of 6-methylpterin from which the free base could be isolated in pure state by acidification. It turned out to be possible, by Taylor's method, to prepare a 2,4-diamino-6-methylpteridine which was fully deuterated on C(7) and partially so on the C(6)-methyl group (see Section 2.3). The final step in the preparation of 7-deutero-6-methylpterin with a methyl group that is either fully deuterated or does not contain any deuterium at all was therefore simply to heat and recrystallize the 2,4-diamino-7-deutero-6-trideuteromethyl(partial)pteridine with 2N-sodium hydroxide or 2N-deuteroxide to give (in excellent yields) 7-deutero-6-methylpterin (2.2) and 7-deutero-6-trideuteromethylpterin (2.4) respectively.

2.3 Synthesis of 2,4-diamino-6-methyl- and 2,4-diamino-7-deutero-6-trideuteromethyl(partial)-pteridines

The unambiguous pteridine synthesis of Taylor and coworkers proved to be very satisfactory for preparation of 2,4-diamino-7-deutero-6-trideuteromethyl(partial)-pteridine. Pilot experiments with non-deuterated reagents gave 2,4-diamino-6-methylpteridine (2.13) in good yields, so deuterated starting materials were used. Perdeutero-
hydroxyiminoacetone (2.21) and aminomalononitrile p-toluenesulphonate (2.17) reacted to give 2-amino-3-cyano-6-deutero-5-trideuteromethylpyrazine 1-oxide (2.24) in which only a small percentage of the deuterium on the methyl group and none of the D(6) had been lost.

During the following deoxygenation by phosphorus trichloride, as much as 60-80% of the deuterium on the methyl group was exchanged depending on the time required for the isolation of the product from the resulting slightly basic aqueous solution. The important thing, however, was that all the deuterium on C(6) was retained after the reduction which thus yielded 2-amino-3-cyano-6-deutero-5-trideuteromethyl(partial)pyrazine (2.30, R¹ partially H). In contrast with the corresponding 3-ethoxycarbonyl derivative (2.34), the deuterated nitrile did condense with guanidine in the presence of methoxide. Furthermore a good yield of 2,4-diamino-6-methylpteridine was obtained in which C(7) had retained completely its deuterium atom, and the 6-methyl group was ~65% deuterated (if a pyrazine of high deuterium content in the methyl group was used for the condensation). The loss of deuterium from the methyl group was not surprising because, as mentioned earlier, all the hydrogen atoms on the methyl group in 6-methylpterin was exchanged in the presence of sodium deuteroxide.

While the above experiments were in progress, an attempt was also made to prepare 2-amino-3-cyano-6-deutero-5-methylpyrazine 1-oxide (2.23) by treating the corresponding 6-ethoxycarbonylpyrazine 1-oxide with deuterotrifluoroacetic acid in the hope that it would hydrolyse and decarboxylate to insert a deuterium atom at C(6).
The method turned out to be unsuccessful because ethyl $\alpha$-nitrosoacetoacetate failed to condense with aminomalonic nitrile $p$-toluenesulphonate under the general conditions for this type of condensation.

2.4 Synthesis of 6,7-dimethylpterin and 6,7-bis(trideuteromethyl)pterin

6,7-Dimethylpterin (2.5) was readily prepared in very good yields by a modified Isay method using 2,4,5-triaminopyrimidin-6(1H)-one and biacetyl in the presence of sodium sulphite. Attempts were made to introduce deuterium atoms in the product by direct deuterium exchange reactions. The methyl protons of the dimethylpterin were, as the methyl protons in 6-methylpterin, barely exchanged by deuterium in deuterated acid. In deuterium oxide containing sodium deuteroxide (2N), on the other hand, the exchange was very much faster, and 6,7-bis(trideuteromethyl)pterin (2.6) of isotopic purity (> 98%) was obtained.

2.5 Catalytic reduction of 6-methyl- and 6,7-dimethyl-pterin, 2,4-diamino-6-methylpteridine and their deuterated derivatives

No concrete rules can be laid down for the reduction of the pteridine nucleus in general. In addition to variations in the reduction of the nucleus when different reducing reagents are used, the position and nature of substituents often play a determining role. Both
chemical and catalytic reduction of pterins and 2,4-diaminopteridines take place mainly in the pyrazine ring because this ring is quite susceptible to reduction, whereas the pyrimidine ring is very difficult to hydrogenate, particularly in alkaline medium. Only a few examples of reduction in the pyrimidine ring of pteridines have been reported. All the reductions carried out in the present work took place in the pyrazine ring and gave 7,8-dihydro or 5,6,7,8-tetrahydro pteridines depending on the duration of the reduction and on the reducing agent. Thus, catalytic reduction of the previously prepared pteridines and their deuterated derivatives in 3N-hydrochloric acid or in trifluoroacetic acid gave high yields of 6-methyl- (2.7), 7-deutero-6-methyl- (2.8), 6-trideuteromethyl- (2.9), 7-deutero-6-trideuteromethyl- (2.10), 6,7-dimethyl- (2.11) and 6,7-bis(trideuteromethyl)- (2.12) 5,6,7,8-tetrahydropterins and 2,4-diamino-6-methyl- (2.15) and 2,4-diamino-7-deutero-6-trideuteromethyl(partial)- (cf.2.16) 5,6,7,8-tetrahydropteridines. Catalytic reduction of the deuterated compounds took place without observable loss of deuterium. If necessary, the products were purified further and isolated as hydrochlorides by dissolving the compounds in a solution of hydrochloric acid in methanol followed by precipitation with ether. The general method for the preparation of tetrahydro derivatives is described in more detail in the Experimental Section, p.115.
2.6 Stereochemistry of the catalytic reduction of 6-methyl- and 6,7-dimethyl-pterin, 2,4-diamino-6-methylpteridine and their deuterated derivatives

2.6.1 Some general considerations regarding the reduction of pteridines

When the planar pyrazine ring is hydrogenated (which occurs in two steps; see later), the relative stereochemistry of the hydrogen atoms that add to C(6) and C(7) can be cis or trans. In reducing the pyrazine ring, new properties of the ring have been introduced, namely chirality and mobility. The latter arises from the possibility of the reduced ring to oscillate between two half-chair conformations (the half-boat conformation being energetically less favourable because of torsional (Pitzer) strain; 11.3 KJ mol⁻¹ for cyclohexene). In spite of the distortion from a normal chair form, C(6) and C(7) are reported to be normally staggered so H(6) and H(7) occupy normal axial (ax) and equatorial (eq) positions. H(5) and H(8), however, are imperfectly staggered and are said to occupy pseudo-equatorial or pseudo-axial positions.

The hydrogen atoms at C(6) and C(7) which have added cis can equilibrate in the following way:

\[ H(6)_{\text{ax}} H(7)_{\text{eq}} \rightleftharpoons H(6)_{\text{eq}} H(7)_{\text{ax}} \]

The position of the equilibrium depends on the number of methyl substituents in the pyrazine ring and on the asymmetry imposed by the amino-oxopyrimidine ring.
\[ R^1 = \text{CH}_3, \ R^2 = \text{H} \] (2.41)
\[ R^1 = \text{CD}_3, \ R^2 = \text{H} \] (2.42)
\[ R^1 = \text{CD}_3, \ R^2 = \text{D} \] (2.43)
\[ R^1 = R^2 = \text{CH}_3 \] (2.44)
\[ R^1 = R^2 = \text{CD}_3 \] (2.45)

\[ R^1 = R^2 = \text{CD}_3 \] (2.45a)
In the case of 6-methyl-5,6,7,8-tetrahydropterin (see equilibrium \(2.41 \rightleftharpoons 2.41a\)), the conformer \((2.41)\) with the methyl group in the equatorial position is energetically favoured. \(^{144}\) In cis-6,7-dimethyl-5,6,7,8-tetrahydropterin, on the other hand, the two conformers \((2.44\) and \(2.44a\)) should be almost equally favoured like they are known to be in the related ring, cis-2,3-dimethyl-1,2,3,4-tetrahydroquinoxaline. \(^{145}\) However, this may not be entirely so in the tetrahydropterin because of the presence of the fused pyrimidine ring.

The following equilibrium can occur, if the hydrogen atoms have added \textit{trans} to C(6) and C(7) in the pteridine:

\[
H(6)_{ax}H(7)_{ax} \rightleftharpoons H(6)_{eq}H(7)_{eq}
\]

It is not possible to distinguish between the two hydrogen atoms on C(7) in 6-methyl-5,6,7,8-tetrahydropterin, so the equilibrium \(2.46 \rightleftharpoons 2.46a\) is identical with system \(2.41 \rightleftharpoons 2.41a\). It is known, however, that conformer \(2.41 (= 2.46)\) with an equatorial methyl group is favoured. On basis of results from temperature studies (\textit{vide infra}), Archer and Mosher\(^ {145}\) concluded that there is a rapid interconversion of the two conformers of \textit{trans}-2,3-dimethyl-1,2,3,4-tetrahydroquinoxaline (down to \(-87^\circ\)), with the equilibrium ratio greatly in favour of the conformer with diequatorial methyl groups. This suggests that the equilibrium \(2.49 \rightleftharpoons 2.49a\) of the conformers of \textit{trans}-6,7-dimethyl-5,6,7,8-tetrahydropterin is shifted to the left in favour of \(2.49\) where CH\(_3\)(6) and CH\(_3\)(7) are in equatorial positions. This is also in accordance with
(2.46) \( R_1 = \text{CH}_3, R_2 = \text{H} \)  
(2.47) \( R_1 = \text{CD}_3, R_2 = \text{H} \)  
(2.48) \( R_1 = \text{CD}_3, R_2 = \text{D} \)  
(2.49) \( R_1 = R_2 = \text{CH}_3 \)  
(2.50) \( R_1 = R_2 = \text{CD}_3 \)  

(2.46a) 
(2.47a) 
(2.48a) 
(2.49a) 
(2.50a)
the cyclohexane system where it is known that an equatorial methyl is favoured in respect to the axial methyl conformer by an enthalpy difference of 7.95 KJ mol\(^{-1}\) (at 25\(^{\circ}\) in the gas phase). But, as Archer and Mosher\(^{145}\) also point out for 2,3-disubstituted tetrahydroquinoxalines, it is not obvious \textit{a priori} that the half-chair form with equatorial substituents would be the favoured conformation in the tetrahydro-pyrazines. It has to be taken into account that, in contrast with cyclohexane which has two 1,3-diaxial interactions for each substituent in the chair conformation, both the CH\(_3\)(6)/H(8) or N-lonepair and the CH\(_3\)(7)/H(5) or N-lonepair diaxial non-bonded interactions are very small (or do not exist) in the half-chair conformation of the tetrahydropyrazine ring in the above compounds. Furthermore, the two remaining CH\(_3\)(6)/N(8) and CH\(_3\)(7)/N(5) diaxial interactions will be with the electron pairs on the nitrogen atoms which are conjugated with the pyrimidine ring. The conformational preference for a non-bonding electron pair on nitrogen in saturated six-membered ring has been reported\(^{146}\) for several systems, but no data from a system strictly comparable with the reduced pteridines in this work appear to be available.

It should be possible, by means of p.m.r. spectroscopy, to answer the question of whether the catalytic addition of hydrogen to C(6) and C(7) in 6-methyl- and 6,7-dimethylpterin and in 2,4-diamino-6-methylpteridine to give the corresponding 5,6,7,8-tetrahydro derivatives occurs with \textit{cis} or \textit{trans} specificity. It seems reasonable to assume that the coupling constant from the p.m.r.
spectra of the reduced pteridines should be of the same order as those obtained from the already mentioned related compounds, 1,2,3,4-tetrahydroquinoxalines. Archer and Mosher\textsuperscript{145} reported that the p.m.r. spectra of \textit{cis}- and \textit{trans}-2,3-dimethyl-1,2,3,4-tetrahydroquinoxaline exhibit a chemical shift difference of 0.5 p.p.m. in the signals from the protons on \textit{C}(2) and \textit{C}(3), adjacent to the nitrogens [compare \textit{C}(6) and \textit{C}(7) in the pteridines]. They attributed this difference to the unequal shielding of axial and equatorial protons resulting from the diamagnetic anisotropy of the carbon-nitrogen single bond. Substitution in the aromatic ring had little effect on these chemical shifts. Aguilera, Duplan and Nofre\textsuperscript{147} also recorded the p.m.r. spectra of \textit{cis}- and \textit{trans}-2,3-dimethyl-1,2,3,4-tetrahydroquinoxaline and of the 2-methyl derivative. From these spectra, they obtained the following coupling constants, which are time-averaged because other conformers could not be excluded entirely from the solutions. For \textit{N}-inversion see p.67.

Table 2.1

<table>
<thead>
<tr>
<th>Coupling constants of 2-methyl- and 2,3-dimethylquinoxaline\textsuperscript{147}</th>
<th>( J ) (in Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{cis}</td>
<td>CoUpling between \textit{H}(2) and \textit{H}(3) (ax-eq): ( \sim 3 )</td>
</tr>
<tr>
<td></td>
<td>Vicinal coupling between \textit{CH}_3 and \textit{H} ( \sim 6 )</td>
</tr>
<tr>
<td>\textit{trans}</td>
<td>Vicinal coupling between \textit{CH}_3 and \textit{H} ( \sim 6 )</td>
</tr>
<tr>
<td></td>
<td>Coupling between \textit{H}(2) and \textit{H}(3): ( \sim 8 ) (predominantly by axial-axial)</td>
</tr>
<tr>
<td></td>
<td>Geminal coupling between \textit{H}(3)\textit{ax} and \textit{H}(3)\textit{eq} ( \sim 11 ) (only in 2-methyltetrahydroquinoxaline)</td>
</tr>
</tbody>
</table>
With the assumption that the p.m.r. spectra of the two reduced ring systems (quinoxalines and pteridines) should be similar and that substitution of hydrogen by deuterium has negligible effect on the conformations in these examples, a distinction between cis and trans addition of hydrogen in pteridines can be made because of the significant difference (~5 Hz) between the vicinal coupling constant for the cis protons (~3 Hz) and the corresponding trans protons (~8 Hz).

In 5,6,7,8-tetrahydropteridines the conformations of the hydrogen atoms at N(5) and N(8) should not affect the conclusions derived from $J$ values about the stereochemistry of the hydrogen atoms at C(6) and C(7). These nitrogen atoms undergo atomic inversion, which is probably quite rapid, and would influence the rate of equilibration of the half-chair conformations of the tetrahydropyrazine rings. Unfortunately the hydrogen atoms on N(5) and N(8) are also readily exchangeable, and information regarding the stereochemistry of the two individual steps in the reduction (see later), i.e. addition of hydrogen to the N(5), C(6) and C(7), N(8) double bonds, would be difficult to obtain.

2.6.2 The p.m.r. spectra and stereochemistry of 6-methyl-, 6-trideuteromethyl- and 7-deutero-6-trideuteromethyl-5,6,7,8-tetrahydropterin hydrochlorides

The p.m.r. spectrum of 6-methyl-5,6,7,8-tetrahydropterin hydrochloride in deuterium oxide at 100 MHz
(see Figure 2.1) is similar to the spectrum recorded in 0.5N-deuterium chloride by Weber and Viscontini.\textsuperscript{144} The two diastereotopic H(7) protons form together with H(6) an ABC type of coupling pattern. Such a pattern consists theoretically of fifteen lines of which three are combination lines. It is, however, only possible to see fourteen lines in the spectrum because one combination line has zero intensity (forbidden transition).\textsuperscript{148-150} For simplicity only the spectral lines from 6-methyl-5,6,7,8-tetrahydropterin in the favoured conformation (2.41) (see later) with the methyl group in the equatorial position is considered. It is therefore necessary in the following to keep in mind that the signals in the p.m.r. spectrum are time-averaged chemical shifts including probably some contribution from the less favoured conformer (2.41a). It is known from cyclohexane, for example, that axial protons appear at a slightly higher field (\(\sim 0.5\) p.p.m.) than equatorial protons.\textsuperscript{149} On this basis, the order of appearance of the proton signals in the p.m.r. spectrum of the tetrahydropterin should be \(H(7)_{eq}\), \(H(7)_{ax}\) and \(H(6)_{ax}\), with \(H(6)_{ax}\) at the highest field because this proton is located next to a slightly electron-donating methyl group and therefore is more shielded than \(H(7)_{ax}\). Surprisingly, this is not so. Viscontini and Weber\textsuperscript{144} showed that \(H(7)_{ax}\) appears at the highest field (see later) reflecting the unequal deshielding effects from N(5) and N(8). The theoretical ABC type of coupling pattern from
4.20
3.86
1.98 p.p.m.

Figure 2.1

P.m.r. spectrum of 6-methyl-5,6,7,8-tetrahydroppterin hydrochloride in 0.5N-DC1/D2O at 100 MHz
H(7)\textsubscript{eq}, H(6)\textsubscript{ax} and H(7)\textsubscript{ax} in the p.m.r. spectrum of tetrahydropterin (6-trideuteromethyl) is shown in Figure 2.2 (which is based on a computer-simulated spectrum from the experimental signal positions; see later). The complicating factor in the 100 MHz p.m.r. spectrum is the further coupling of H(6) with the Ζ(6)-methyl group which makes the theoretical spectrum of H(6) consist of sixteen lines.

From the 100 MHz p.m.r. spectrum of 6-deutero-6-methyl-5,6,7,8-tetrahydropterin\textsuperscript{*} (see Figure 2.3), Weber and Viscontini\textsuperscript{144} assigned the AB pattern from the two Ζ(7)-protons and measured their coupling constant (\(J_{7,7} = 13\) Hz, from spectrum recorded in 0.5\textsubscript{N}-DC\textsubscript{1}/D\textsubscript{2}0). They further deduced the vicinal coupling constants between H(6) and the two Ζ(7)-protons in 6-methyl-5,6,7,8-tetrahydropterin by computer-simulation [\(J_{6,7}(\text{cis}) = 3\) Hz and \(J_{6,7}(\text{trans}) = 10.0\) Hz].

The chemical shifts of H(7)\textsubscript{ax} and H(7)\textsubscript{eq} in 0.5\textsubscript{N}-DC\textsubscript{1}/D\textsubscript{2}0 were reported to be 4.05 p.p.m. [H(7)\textsubscript{ax}] and 4.43 p.p.m. [H(7)\textsubscript{eq}]. The signals from H(6)(δ 4.34 p.p.m.) were almost on top of those from H(7)\textsubscript{eq}.

Attempts were made in the present work to convert the ABC pattern in the p.m.r. spectrum of 6-methyl-5,6,7,8-tetrahydropterin into a first-order spectrum by

Footnote: * The spectrum was recorded in 1.3\textsubscript{N}-NaOD/D\textsubscript{2}0 instead of 0.5\textsubscript{N}-DC\textsubscript{1}/D\textsubscript{2}0. Weber and Viscontini reported that the change of solvent caused an upfield shift but hardly any change in the coupling constants.
Figure 2.2 Theoretical ABC type (AA'B) coupling pattern in the p.m.r. spectrum of 6-methyl-5,6,7,8-tetrahydropterin (based on a computer-simulated spectrum from the experimental signal positions; $J$ values are in Hz)
Figure 2.3

P.m.r. spectrum of
6-deutero-6-methyl-5,6,7,8-tetrahydropterin
in 1.3N-NaOD/D₂O at 100 MHz.¹⁴⁴
varying the solvent (e.g. CF₃COOH, D₂SO₄, (CD₃)₂SO, (CD₃)₂NCDO and DCI), but these all failed to simplify the spectrum. In a further attempt to obtain a first-order pattern of p.m.r. signals, the spectrum was recorded at 270 MHz (see Figure 2.4.a). The signal pattern for H(6) and H(7) at this field is slightly different from the one at 100 MHz (Figure 2.1), but there appears to be very little dispersion between H(6) and H(7)_eq. The 7-axial proton (δ 3.86 p.p.m.) and the 7-equatorial proton (δ 4.20 p.p.m.) are clearly separated and have a coupling constant of -13 Hz. Their coupling constant with H(6) could be measured (J_{7ax}^6ax = 10.0 Hz and J_{7eq}^6ax = 3 Hz; see Figure 2.4.a and Table 2.2).

The magnitudes of the measured coupling constants support the theory that the tetrahydropyrazine ring exists in a half-chair conformation (2.41) or equilibrating half-chair conformations (2.41 ~ 2.41a) where the ratio of the conformers lies in favour of the one (2.41) with the methyl group in the equatorial position. The predominance of the latter conformer is indicated by the large coupling constant of 10.0 Hz (axial/axial coupling) which is of the same order as the one reported for the related trans-2,3-dimethyl-1,2,3,4-tetrahydroquinoxaline system.¹⁴⁷

The p.m.r. spectrum of 6-trideuteromethyl-5,6,7,8-tetrahydropterin hydrochloride in deuterium oxide at 100 MHz (Figure 2.5) and 270 MHz (Figure 2.4.b) are also similar to each other, with a very small difference in chemical shifts between H(6) and H(7)_eq. The signal pattern is simplified because of the missing coupling
Figure 2.4

P.m.r. spectra of (a) 6-methyl-5,6,7,8-tetrahydropterin hydrochloride in D₂O at 270 MHz; (b) 6-trideuteromethyl-5,6,7,8-tetrahydropterin hydrochloride in D₂O at 270 MHz; (c) cis- and trans-7-deutero-6-trideuteromethyl-5,6,7,8-tetrahydropterin hydrochloride in D₂O at 270 MHz.
Table 2.2

<table>
<thead>
<tr>
<th>Compound</th>
<th>solvent</th>
<th>CH₃</th>
<th>H(6)</th>
<th>H(7)</th>
<th>CHD₂</th>
<th>D₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-Trideuteromethyl (2.47)</td>
<td>D₂O</td>
<td>-</td>
<td>4.19₃</td>
<td>4.19₈</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6-Methyl (2.41)</td>
<td>D₂O</td>
<td>-</td>
<td>(as above and J 6.4)</td>
<td>(as above and J 6.4)</td>
<td>CH₃(6) 1.98 (J 6.4)</td>
<td>D₂O</td>
</tr>
<tr>
<td>6-Methyld</td>
<td>D₂O</td>
<td>-</td>
<td>4.43</td>
<td>10.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>cis-7-Deutero-6-trideuteromethyl (2.48)</td>
<td>D₂O</td>
<td>-</td>
<td>4.20</td>
<td>9.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>cis-7-Deutero-6-trideuteromethyl partial</td>
<td>D₂O</td>
<td>-</td>
<td>4.20</td>
<td>9.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2,4-Diamino-5,6,7,8-tetrahydropteridine hydrochloride</td>
<td>D₂O</td>
<td>-</td>
<td>2.03 (J 6.3)</td>
<td>2.03 (br. d.)</td>
<td>0.5N DCl</td>
<td></td>
</tr>
<tr>
<td>6-Methyl (2.15)</td>
<td>D₂O</td>
<td>-</td>
<td>4.19e</td>
<td>10.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>trans-7-Deutero-6-trideuteromethyl (partial)</td>
<td>D₂O</td>
<td>-</td>
<td>3.84 (J 10.2)</td>
<td>3.84 (J 10.2)</td>
<td>CHD₂(6) 2.03 (br. d.)</td>
<td>D₂O</td>
</tr>
<tr>
<td>cis-7-Deutero-6-trideuteromethyl partial</td>
<td>D₂O</td>
<td>-</td>
<td>4.20</td>
<td>-</td>
<td>CHD₂(6) 2.03 (br. d.)</td>
<td>D₂O</td>
</tr>
<tr>
<td>5,6,7,8-Tetrahydropterin hydrochloride</td>
<td>0.5N DCl</td>
<td>-</td>
<td>1.87 (J 6.7)</td>
<td>1.87 (J 6.7)</td>
<td>D₂O</td>
<td></td>
</tr>
<tr>
<td>cis-6,7-Dimethyl (2.44)</td>
<td>CH₃(6)</td>
<td>-</td>
<td>1.79 (J 6.7)</td>
<td>1.79 (J 6.7)</td>
<td>D₂O</td>
<td></td>
</tr>
<tr>
<td>cis-6,7-Dimethyl partial (2.45)</td>
<td>CH₃(6)</td>
<td>-</td>
<td>1.87 (J 6.7)</td>
<td>1.87 (J 6.7)</td>
<td>D₂O</td>
<td></td>
</tr>
<tr>
<td>trans-6,7-Dimethyl (2.49)</td>
<td>CH₃(6)</td>
<td>-</td>
<td>1.95 (J 6.8)</td>
<td>1.95 (J 6.8)</td>
<td>D₂O</td>
<td></td>
</tr>
</tbody>
</table>

---

a Concentration 20 mg in 0.5 ml; tetramethylsilane as external standard.
b Computer-simulated spectrum from the experimental signal positions (by M.J. Whittaker), error ± 0.1 Hz.
c Data at 270 MHz are almost identical with these although the spectrum appears simpler (see Figure 2.4).
d From ref. 145.
e Complex multiplet.
f Assignments taken from ref. 151.
g,h Tentative, assignments may be reversed.
between H(6) and CD₃(6) and the only very small coupling between H(6) and CD₃(6). This means that instead of having sixteen lines from H(6), it is reduced to a much clearer four-line envelope. The quartet for H(7)eq is not clearly visible and forms the lowest-field quartet from H(7)eq. It was, however, disappointing to find that the four signals from H(7) almost disappear in the band envelope. The p.m.r.
spectrum was therefore computer-simulated (mainly computed by Mr. J. Whitaker, Research School of Chemistry, Australia) and by doing so the quartets for each
of the three protons H(6), H(7)eq, and H(7)ax became
more easily distinguishable. The simulated spectrum and the one obtained from the peak (see Table 2.2) are in good agreement. The computed coupling constants are
a(6,7eq) = 9.0 Hz and a(6,7ax) = 5.0 Hz, the latter 3.4 Hz
larger than for the non-deuterated compound (Table 2.2). The 1H spectrum of 6-trideuteromethyl-5,6,7,8-
tetrahydropterin hydrochloride in D₂O at 100 MHz
Figure 2.5

P.m.r. spectrum of 6-trideuteromethyl-5,6,7,8-
tetrahydropterin hydrochloride in D₂O at 100 MHz

<table>
<thead>
<tr>
<th>δ</th>
<th>4.20</th>
<th>3.86</th>
<th>p.p.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>δ</td>
<td>4.20</td>
<td>3.86</td>
<td>p.p.m.</td>
</tr>
</tbody>
</table>

It is now possible, with the knowledge of the above
assignments, to determine the stereochemistry addition
between this work and tetrahydropterin. The spectrum of the tetrahydro derivative
between H(6) and CH₃(6) and the only very small coupling between H(6) and CD₃(6). This means that instead of having sixteen lines from H(6), these are now reduced to four. The quartet from H(7)ₐₓ in conformer 2.42 is still clearly visible and so is the downfield quartet from H(7)ₑₜ. It was, however, disappointing to find that the four signals from H(6) almost disappears in a band envelope. The 270 MHz p.m.r. spectrum was therefore computer-simulated (kindly computed by Mr M.J. Whittaker, Research School of Chemistry, ANU) and by doing so the quartets for each of the three protons, H(6), H(7)ₐₓ and H(7)ₑₜ, became clearly distinguishable. The simulated spectrum and the data obtained from this (see Table 2.2) indicate that these spin systems are more like an AA'B system than an ABC system. The computed coupling constants (\(Jₐₓ ₐ_x^{7} = 3.6\ Hz, \ Jₐ_x ₐ_x^{6} = 9.0\ Hz\) and \(Jₐ_x ₐ_x^{7} = -14.2\ Hz\)) are all very close (within ±0.1 Hz) to the values measured directly from the spectrum at 270 MHz but not at 100 MHz (for comparison see Table 2.2). The former spectrum is closer to a first-order spectrum with regard to H(7)ₐₓ and H(7)ₑₜ but the signals from H(6) are still too broad to be assigned by inspection. The difference in \(J\) values between this work and that reported¹⁴⁴ (\(\sim Δ 0.2\ p.p.m.\)) may be partly due to the effect of the solvent on the equilibrium 2.42 ≡ 2.42a.

It is now possible, with the knowledge of the above assignments, to determine the stereochemistry of addition of hydrogen to C(6) and C(7) in 7-deutero-6-trideutero-methylpterin. The spectrum of the tetrahydro derivative
(hydrochloride; see Figure 2.4.c) consists of two doublets (\( J \approx 9.0 \) Hz) and a singlet inside the downfield doublet. The chemical shifts of these signals, when compared with those of 6-trideuteromethyl-5,6,7,8-tetrahydropterin hydrochloride (see the lined up spectra (b) and (c) in Figure 2.4) show that the upfield doublet must be from \( H(7)_{ax} \) and that the downfield doublet is from \( H(7)_{eq} \). The two doublets can only be from the trans-isomer in the predominant conformation 2.48 in which the trideuteromethyl group is equatorial. The singlet at 4.19 p.p.m. comes from the cis-isomer in the predominant conformation 2.43 with almost similar chemical shifts for \( H(6)_{ax} \) and \( H(7)_{eq} \). The slightly broadened singlet is not centred between the downfield doublet. This can be explained if the equilibria of the conformers 2.48 \( \rightleftharpoons \) 2.48a and 2.43 \( \rightleftharpoons \) 2.43a are slightly different in the two isomers. The difference could be caused by the effect of the deuterium atom on the conformational equilibria, or a deuterium isotope effect on the chemical shift of the geminal proton, but is probably not of steric origin because of the pseudo-axial and pseudo-equatorial positions of the protons on N(5) and N(8), and the negligible difference in the space demanding properties of a hydrogen and deuterium atom. The \( \text{cis:trans} \) ratio calculated from the integrals of the signals in the p.m.r. spectrum of 7-deutero-6-trideuteromethyl-5,6,7,8-tetrahydropterin hydrochloride is 0.8:1.0.
2.6.3 The p.m.r. spectrum and stereochemistry of 2,4-diamino-6-methyl-5,6,7,8-tetrahydropteridine and of 2,4-diamino-7-deutero-6-trideuteromethyl(partial)-5,6,7,8-tetrahydropteridine hydrochlorides

The p.m.r. spectrum of 2,4-diamino-6-methyl-5,6,7,8-tetrahydropteridine hydrochloride in deuterium oxide at 100 MHz (see Figure 2.6) is quite similar to that of 6-methyl-5,6,7,8-tetrahydropterin hydrochloride. The $\zeta(7)$-protons could be assigned by inspection of the AA'BB pattern of signals. The protons $H(7)_{ax}$ and $H(7)_{eq}$ have chemical shifts of 3.84 and 4.20 p.p.m. respectively (for comparison with values from the tetrahydropterin, see Table 2.2 where also the measured coupling constants are reported).

The p.m.r. spectrum of 2,4-diamino-7-deutero-6-trideuteromethyl(partial)-5,6,7,8-tetrahydropteridine hydrochloride in deuterium oxide at 100 MHz (see Figure 2.7) is similar to the 270 MHz spectrum of the corresponding cis- and trans-7-deutero-6-trideuteromethyl-5,6,7,8-tetrahydropterin (Figure 2.4.c) which indicates that catalytic reduction of the diaminopteridine also gives a mixture of cis and trans isomers. The downfield doublet at 4.19 p.p.m. from $H(6)_{ax}$ in the trans-isomer is almost covered by the singlet from $H(6)_{ax}$ and $H(7)_{eq}$ in the cis-isomer. The cis:trans ratio is ca. 1:1.
Figure 2.6

P.m.r. spectrum of 2,4-diamino-6-methyl-5,6,7,8-tetrahydropterin hydrochloride in D₂O at 100 MHz
Figure 2.7

P.m.r. spectrum of 2,4-diamino-7-deutero-6-trideuteromethyl-(partial)-5,6,7,8-tetrahydropterin hydrochloride in D₂O at 100 MHz
2.6.4 The p.m.r. spectra and stereochemistry of 6,7-dimethyl- and 6,7-bis(trideuteromethyl)-5,6,7,8-tetrahydropterin hydrochlorides

The p.m.r. spectrum of 6,7-dimethyl-5,6,7,8-tetrahydropterin hydrochloride in deuterium oxide at 100 MHz (Figure 2.8.a) has eleven lines (theory: sixteen lines) for H(6) and H(7). The anisotropic shift between the two methyl doublets [CH$_3$(6) at 1.79 p.p.m. and CH$_3$(7) at 1.87 p.p.m.] could be due to the higher basicity of N(5) compared with N(8). The coupling constants for the methyl doublets (6.7 Hz) are almost identical, making the vicinal coupling constants for H(6) and H(7) (3.1 Hz) observable. The small coupling constant corresponds to the literature value of 2.7 Hz for axial-equatorial coupling between two vicinal hydrogens, H(2) and H(3), in 2,3-dimethyl-1,2,3,4-tetrahydroquinoxaline, and suggests that the configuration is $cis$. This configuration is consistent with the half-chair conformation (2.44) or equilibrating half-chair conformations (2.44 $\equiv$ 2.44a), as has previously been postulated for such systems (see p. 63). While this work was in progress, Weber and Viscontini reported that 6,7-dimethyl-5,6,7,8-tetrahydropterin hydrochloride formed by catalytic reduction in the trifluoroacetic acid, was the $cis$ isomer. They stated that "die erhaltenen Produkte [6,7-dimethyl- and

Footnote: *Translation: "the products show the characteristics of one stereochemical compound (sharp melting point, p.m.r. spectrum of a single substance)...."
Figure 2.8

P.m.r. spectra of (a) cis-6,7-dimethyl-5,6,7,8-tetrahydropterin hydrochloride in D₂O at 100 MHz; (b) cis-6,7-bis(trideuteromethyl)-5,6,7,8-tetrahydropterin hydrochloride in D₂O at 100 MHz; (c) cis- and trans-6,7-dimethyl-5,6,7,8-tetrahydropterin hydrochloride in 0.5N-DCl/D₂O at 100 MHz.
5,6,7-trimethyl-5,6,7,8-tetrahydropterin weisen jedoch die Merkmale von stereochemisch einheitlichen Substanzen auf (enges Schmelzpunktsintervall, einheitliche $^1$H-NMR.-Spektren)..." and claimed on this basis that only one diastereomer was present, in spite of the fact that tetrahydropterin hydrochlorides decompose above 240°. Also, the envelope of the signals for cis H(6) and H(7) could conceivably be masking signals from any contaminating trans-isomer which would only be separated 0.5 p.p.m. from the signals of the cis-isomer. 147 Weber and Viscontini further assumed that the C(7)-methyl group is equatorially oriented because N(8) in 6,7-dimethyl-5,6,7,8-tetrahydropterin is less reactive than N(8) in the corresponding 6-methyl derivative, probably due to steric hindrance. In all their N(5)-acyl derivatives they observed an anisotropic shift of the C(6)-methyl group, and therefore assumed that this group was in the axial position. Also, from their well resolved p.m.r. spectrum in 0.5N-DCl/D$_2$O (the H(6) and H(7) signals were separated by ~0.3 p.p.m.), Weber and Viscontini measured a coupling constant, $J_{6,7}$, of 3 Hz (corresponding to the above 3.1 Hz). On the basis of the Karplus-relationship between dihedral angles and coupling constants of vicinal protons, 152 and on the assumption that the conformation of the reduced pyrazine ring is half-chair, they again concluded that the coupling was from axial-equatorial vicinal protons in the cis-configuration. This did not exclude the
possibility of a contaminating trans-stereoisomer with trans-diaxial methyl groups, since the diequatorial H(6) and H(7) would have a small $J$ value as well.

The present work confirmed the absence of the trans-isomer in both crude and purified samples. This was demonstrated by the spectrum of 6,7-bis(trideuteromethyl)-5,6,7,8-tetrahydropterin hydrochloride and of a prepared mixture of cis- and trans-6,7-dimethyl-5,6,7,8-tetrahydropterin hydrochloride.

In 6,7-bis(trideuteromethyl)-5,6,7,8-tetrahydropterin hydrochloride, the coupling constants between the deuterium atoms of the methyl groups and H(6) and H(7) are too small to be observed and a simplified spectrum is obtained (Figure 2.8.b). This spectrum shows only two doublets, from which the coupling constant between H(6) and H(7) can be readily measured. The $J$ value (3.1 Hz) and the chemical shifts of the two doublets [4.34 p.p.m. for H(6) and 4.43 p.p.m. for H(7)\(^*\)] are consistent with the values derived from the multiplet of the non-deuterated isomer (see Table 2.2). There is no evidence for the presence of signals from a contaminating second isomer.

The above deductions from the present work and those reported\(^{151}\) were made only from the knowledge that the coupling constant between H(6) and H(7) is small. However, the cis-configuration of 6,7-dimethyl-5,6,7,8-tetrahydropterin from catalytic reduction was confirmed beyond doubt by obtaining a ca. 1:1 mixture of cis- and trans- tetrahydro derivatives (hydrochlorides) by reducing 6,7-dimethylpterin with sodium in ethanol.

\(^*\)assignments taken from ref.151.
Chromatographic separation of the mixture has not yet been achieved. However, the p.m.r. spectra of the isomers are clearly separated (see Figure 2.8.c) and the coupling constant between H(6) and H(7) in the trans-isomer is significantly larger (ΔJ ≈ 5 Hz) than that in the cis-isomer (see Table 2.2). The coupling constant between H(6)_{ax} and H(7)_{ax} (8.3 Hz) in trans-6,7-dimethyl-5,6,7,8-tetrahydropterin is of the same order as J_{2,3} obtained from trans-2,3-dimethyl-1,2,3,4-tetrahydroquinoxaline which (in this case) justifies the comparison between reduced pteridines and reduced quinoxalines.

Catalytic addition of hydrogen to C(6) and C(7) in 6,7-dimethylpterin (in 3N-hydrochloric acid and trifluoroacetic acid) is therefore entirely cis. This was later confirmed further by Bieri and Viskontini\textsuperscript{143} who published an X-ray analysis of 5,6,7-trimethyl-5,6,7,8-tetrahydropterin dihydrochloride monohydrate (prepared from 6,7-dimethyl-5,6,7,8-tetrahydropterin) which showed that H(6) and H(7) are in the cis-configuration with the C(6)-methyl group in the axial position in the crystal.

2.6.5 Some considerations concerning the stereospecificity of catalytic reduction of 6-methyl- and 6,7-dimethyl- pterins

It was found that the catalytic reduction of both 6-methylpterin and 6,7-dimethylpterin occurs in two steps: addition of one molecule of hydrogen across the 7,8-double bond followed by addition of a second molecule of hydrogen across the 5,6-double bond. This was confirmed
in the present work by withdrawing aliquots from the reaction mixture during catalytic reduction, and estimating the ratios of unreduced pterin to dihydro derivative and to tetrahydro derivative by p.m.r. and u.v. spectroscopy. The data obtained in connection with the catalytic reduction of 6-methylpterin (see Table 2.3) showed clearly that all the pterin was reduced to the 7,8-dihydro derivative (after uptake of 1 mol. equiv. of hydrogen) before any significant amount of tetrahydropterin was formed. The formation of 7,8-dihydropterin was confirmed by evaporation of an aliquot withdrawn after absorption of 1 mol. equiv. of hydrogen, and formation of the dithionite salt which proved to be identical with authentic 6-methyl-7,8-dihydropterin dithionite. It can also be seen from Table 2.3 that addition of hydrogen across the 7,8-double bond in 6,7-dimethylpterin was slower than the corresponding addition in 6-methylpterin. The intermediate was shown to be 6,7-dimethyl-7,8-dihydropterin by aeration of a fully reduced sample, giving rise to a product with u.v. properties similar to those obtained from the sample withdrawn after absorption of 1.5 mol. equiv. of hydrogen. The p.m.r. spectra were also consistent with these findings. Aeration of 6,7-dimethyl-5,6,7,8-tetrahydropterin is known to furnish the 7,8-dihydro derivative. 153

The difference in the stereospecificity between the catalytic additions of hydrogen to 6,7-dimethylpterin
Table 2.3

Ratios of 6-methylpterin (Me-P) to 6-methyl-7,8-dihydropterin (Me-DHP) to 6-methyl-5,6,7,8-tetrahydropterin (Me-THP) and of 6,7-dimethylpterin (Me₂-P) to 6,7-dimethyl-7,8-dihydropterin (Me₂-DHP) to 6,7-dimethyl-5,6,7,8-tetrahydropterin (Me₂-THP) during catalytic reduction.

<table>
<thead>
<tr>
<th>H₂ absorbed (moles)</th>
<th>Me-P</th>
<th>Me-DHP</th>
<th>Me-THP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4</td>
<td>0.7  : 1.0 : 0.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.9</td>
<td>0.1  : 1.0 : 0.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>0.0  : 1.0 : 0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>0.0  : 0.0 : 1.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Me₂-P</th>
<th>Me₂-DHP</th>
<th>Me₂-THP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>0.4     : 1.0 : 0.4</td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>0.1     : 0.8 : 1.0</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>0.0     : 0.0 : 1.0</td>
<td></td>
</tr>
</tbody>
</table>
and to 6-methylpterin deserves some comment. The stereospecificity of cis-addition in 6,7-dimethylpterin can be explained by adsorption of the molecule on the reduced catalyst and the addition of one molecule of hydrogen across the 7,8-double bond. Then one of two things can happen: (1) the dihydro substrate is held on the catalyst and a second molecule of hydrogen is added stereospecifically, or (2, which is more likely) the dihydro substrate is desorbed and then re-adsorbed stereospecifically because of the encumbrance of the C(7)-methyl group (i.e. structure 2.52 would be more favoured in the transition state than structure 2.51 which would lead to trans-addition).

In the case of 6-methylpterin, the situation is different. After the 7,8-dihydro compound is formed it must be released into the solution and re-adsorbed on the catalyst, almost randomly because of lack of steric hindrance near C(7), and then it is reduced further across the 5,6-double bond.

The catalytic reduction of 2,4-diamino-6-methylpterin is similarly not stereospecific.
2.2 Experimental

Elemental analyses were determined by the Australian National University Analytical Service Unit. Values for hydrogen and nitrogen were determined by the conventional C and N methods which gave the total H and N respectively. Values for carbon were determined by the weight of the Pd formed in the combustion of the sample with HgO. When amino acids were used as the starting material, the ratios of the HDO peak to be 50 larger than that of the HDO peak. The nitrogen values, obtained from the 15N analyses, were often too low, so it was necessary to use the conventional Flasch-Bues system. Nitrogen impurities were measured in Pyrex cylinders and are uncorrected. Proton spectra were obtained on a Varian T60 spectrometer, and 270 MHz spectra were measured on an AI, NO instrument.
Elemental analyses were determined by the Australian National University Analytical Service Unit. Values for hydrogen and deuterium were obtained by the conventional C and H methods which gave the total H+D percentages as determined by the weight of H$_2$O + HOD formed. For smaller quantities (0.5 mg) a 185B Hewlett-Packard C, H and N analyser was used in which H+D values were determined from the H$_2$O + HOD formed, by g.l.c. (thermal conductivity detector). When Armarego, Milloy and Pendergast used glycine and [2-^2$H_2$]glycine as standards in the latter method, they found the area of the HOD peak to be 5% larger than that of the H$_2$O peak. The determined values of H+D in both methods were calculated from the weights or areas of H$_2$O + HOD produced on the assumption that the H : D atomic ratio was that of the compound analysed. Unless these corrections were made, the hydrogen analyses were unsatisfactory. The nitrogen values, obtained from the 185B analyser, were often 1-2% too low, so it was necessary to use the conventional Preq-Dumas method. Melting points (m.p.) were measured in 'Pyrex' capillaries and are uncorrected. The 60 MHz spectra were obtained on a Varian T-60A spectrometer (at 35°C) and the 100 MHz spectra on a Varian HA 100 spectrometer (kindly run by Mr Bela Paal, Research School of Chemistry, ANU) with tetramethysilane (or sodium 3-(trimethylsilyl)-propanesulphonate for aqueous solutions) as internal standard or tetramethylsilane as external standard, and locked on these standards (δ = 0 p.p.m.). 270 MHz spectra were measured on a Bruker HFX-270 spectrometer by the National NMR Centre (Dr A.J. Jones). $J$ values are in Hz. Unless otherwise stated the p.m.r. spectra were recorded on the 60 MHz machine. Mass spectra (by Dr J.K. MacLeod and staff) were measured on an AEI MS9 instrument. The i.r. spectra (KBr discs
for solids and films for liquids) were obtained on a Pye Unicam SP1000 spectrometer, and the assignments (in cm$^{-1}$) are tentative. The u.v. spectra were measured on a Unicam SP1800 or SP800 spectrophotometer. All the recorded solutions were buffered. Relative intensities are given in brackets after $\lambda_{\text{max}}$. All extracts were dried with anhydrous sodium sulphate and evaporations were carried out in a Büchi evaporator below 30°C and at ca. 18 mmHg, unless otherwise stated. Temperatures are in degrees Celsius (centigrades). Deuterium oxide (>99.9%) was purchased from The Australian Atomic Energy Commission, Lucas Heights, N.S.W. The following abbreviations are used: DMSO for dimethyl sulphoxide-$d_6$, br for broad, str for stretch, s for strong, w and vw for weak and very weak respectively, sh for shoulder, and mw for molecular weight.

**Hydroxyiminoacetone.** Ethyl acetoacetate (29.8 g) and potassium hydroxide (14.0 g; 1.09 mol.equiv.) were dissolved in water (500 ml) and a solution of sodium nitrite (16.5 g; 1.04 mol.equiv.) in water (70 ml) was added dropwise. After standing for 36 h, the solution was acidified (to \$pH 4\$) with $\n$-hydrochloric acid, and extracted with ether (6 x 25 ml) after salting with sodium sulphate. The combined ether extracts were washed with water (5 x 5 ml) and dried. Evaporation of the solvent left white yellow crystals of hydroxyiminoacetone (5.8 g; 15%; lit.,$^{134}$ yield 56.5%). Two thirds of these crystals melted at 46-48°C and one third at 92-103°C (lit.,$^{155}$ m.p. 65°C) due to a mixture of isomers as shown by p.m.r.:
δ(DMSO) isomer A, 7.42 (1H, s, CH) and 2.24 (3H, s, CH3); isomer B, 7.59 (1H, s, CH) and 1.90 (3H, s, CH3); νmax. 3250 (br, OH), 3000-2900 (CH str), 1705 (CO), 3000-2900 (CH str), 1705 (CO), 1665 (C=N), 1455, 1375, 1240, 1020 and 1000. The synthesis was repeated twice and all the data are tabulated below.

<table>
<thead>
<tr>
<th>Amount (moles)</th>
<th>Yield (%)</th>
<th>M.p.(°C)</th>
<th>Isomer A</th>
<th>Isomer B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.23</td>
<td>15</td>
<td>46-48 (2/3)</td>
<td>4 : 1</td>
<td></td>
</tr>
<tr>
<td>0.46</td>
<td>63</td>
<td>92-103 (1/3)</td>
<td>16 : 1</td>
<td></td>
</tr>
<tr>
<td>0.92</td>
<td>55</td>
<td>52-55</td>
<td>100 : 1</td>
<td></td>
</tr>
<tr>
<td>Sublimed at 50°/0.1 mmHg</td>
<td>65-66</td>
<td>100% A</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Standing for 24-36 h before acidification was necessary for complete saponification of the ester. Addition of sodium nitrite just before (10 min) acidification did not cause any decrease in the yield. With later syntheses of perdeuterated hydroxyiminoacetone in mind, which would involve use of expensive deuterated reagents in deuterium oxide, the preceding method was studied with two modifications incorporated: (a) a decrease (~ 50%) in the amount of water and (b) use of sodium hydroxide (instead of potassium hydroxide) formed by addition of sodium to water. The reactions were carried out under nitrogen in order to prevent ignition of the liberated hydrogen and, in later syntheses using deuterated reagents, to avoid exchange of D2O by H2O in the air. The changes were generally found to increase the yield (from 57 to 90%) of high-purity hydroxyiminoacetone.

Attempts to prepare hydroxyiminoacetone by the method of Küster were unsuccessful.155 Acetone (7.3 ml; 5.8 g) was mixed with glacial acetic acid (30 g) and cooled to 0°. A solution of sodium
nitrite (15 g; 2.2 mol.equiv.) in water (25 ml) was added with stirring over a period of 10 min, and stirring was continued for 90 min. A water–ether mixture (1:1; 100 ml) was added and the two layers separated. The ether phase was washed with water (2 x 10 ml) and dried. Evaporation of the solvent left no residue. The synthesis was repeated twice with varying reaction times (from 0.75 to 1.5 h) and exhaustive extraction, but all were unsuccessful.

**D/H Exchange in Hydroxyiminoacetone**

In D$_2$O: Hydroxyiminoacetone (40 mg) was dissolved in DMSO (0.5 ml) and stored in an n.m.r. tube at 35°. No changes in the p.m.r. spectrum were observed immediately after shaking with a few drops of D$_2$O. On standing, however, the following proton intensities were measured:

<table>
<thead>
<tr>
<th>Isomer A</th>
<th>Isomer B</th>
</tr>
</thead>
<tbody>
<tr>
<td>t (days)</td>
<td>CH (δ 7.42)</td>
</tr>
<tr>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>4</td>
<td>0.6</td>
</tr>
<tr>
<td>6</td>
<td>0.5</td>
</tr>
<tr>
<td>12</td>
<td>0.4</td>
</tr>
</tbody>
</table>

The B isomer, formed during the exchange, is probably due to syn–anti isomerism.

In NaOD: When hydroxyiminoacetone (44 mg) was dissolved in N-NaOD (0.6 ml; 1.2 mol.equiv.) and stored at 35°, the following proton intensities were measured: CH/CH$_3$ (time in min): 1.0/3.0 (0), 1.0/1.9 (5), 1.0/1.8 (10), 1.0/1.5 (15), 0.9/0.6 (30), 0.8/0.1 (90) and 0.7/0.0 (100).

The time required for 50% exchange ($t_1$) in the methyl group was about 15 min.
Perdeuterated hydroxyiminoacetone.—Ethylacetoacetate (23.8 g) was added to a solution of sodium (4.6 g; 1.1 mol equiv.) in deuterium oxide (300 ml) followed by sodium nitrite (13.2 g), and set aside for 24 h. The reaction was carried out under nitrogen. The mixture was acidified to pH 1 with 10% deuterium chloride/D$_2$O, extracted thoroughly with ether and dried. Evaporation gave the perdeuterated oxime (14.5 g; 87%) which was sublimed at 50° and 0.5 mmHg, and had m.p. 65-66°; the p.m.r. spectrum (in DMSO) showed no peak (δ 7.42) for CH and the methyl signal (δ 2.24) was very weak, and slightly broadened because of geminal H-D coupling; v$_{\text{max}}$, 3300-3200 (OH), 3000-2800 (CH str), 1715 (CO), 1660 (C=N), 1445, 1300, 1030 and 980 (Found: C, 39.7; H+D, 10.0; N, 15.2. C$_3$H$_4$D$_4$NO$_2$ requires C, 39.55; H+D, 9.95; N, 15.4%); m/z 92 (7%, C$_3$D$_2$NO$_2^{2+}$), 91 (100%, C$_3$HD$_4$NO$_2^{2+}$), 90 (24%, C$_3$H$_2$D$_3$NO$_2^{2+}$), 89 (7%, C$_3$H$_3$D$_2$NO$_2^{2+}$), 88 (4%, C$_3$H$_4$DNO;+), and 87 (0).

1-Deutero-1-hydroxyiminoacetone.—t-Butyl α-nitrosoacetoacetate (187 mg) was cooled in ice, and deuterotrifluoroacetic acid (230 mg; 2.0 mol equiv.) was added. No effervescence occurred so the mixture was warmed to 80° for a few minutes. Still no visible effervescence was observed. The p.m.r. spectrum of the residue after evaporation of CF$_3$COOD showed that 1-deutero-1-hydroxyiminoacetone had been formed in ca. 33% yield. If the reaction mixture, before evaporation, was heated on a steam bath for 30 min the yield was increased to 60%, and after storing the mixture overnight at room temperature, only 6% of the starting material was unreacted. The deuterooxime had m.p. 64-65° (after sublimation at 50° and 0.1 mmHg); the CH=NOH signal was absent in the p.m.r. spectrum. The i.r. spectrum was almost identical with that of non-deuterated hydroxyiminoacetone.
**D/H Exchange in perdeuterated hydroxyiminoacetone.**— Sodium hydroxide (~ 25 mg) was added to an n.m.r. tube containing perdeuterated hydroxyiminoacetone (40 mg) dissolved in DMSO. The p.m.r. spectrum was recorded at times ranging from 5 min to 4 days. These spectra showed that the deuterium atoms in the trideuteromethyl group exchanged rapidly ($t_1 = \sim 3$ h), and that on standing the B isomer was gradually formed, A isomer/B isomer (time in days); 1.0/1.2 (1), 1.0/1.3 (2) and 1.0/1.6 (4). An increase in the number of methyl signals after standing suggested, however, that much self condensation had taken place. Also, 2NDCl caused self condensation. The p.m.r. signal from the methyl group altered to a number of weaker methyl signals during less than 6 h storing at 35\(^\circ\).

**Ethyl \(\alpha\)-nitrosoacetoacetate.**— Ethyl acetoacetate (45 g) was mixed with acetic acid (44 ml) and cooled to \(-5^\circ\). A solution of sodium nitrite (22 g; 5.5 mol-equiv.) in water (30 ml) was added slowly (~ 1 h) keeping the temperature between 0\(^\circ\) and 10\(^\circ\). The solution was stirred further (15 min) at 0\(^\circ\), diluted with water (184 ml) and stirring was continued at room temperature overnight. Extraction with ether gave the nitroso ester (57.6 g, crude); b.p. 114-116\(^\circ\) at 1.5 mmHg. \(\delta\) (DMSO) 4.25 (2H, q, \(J 7\), CH\(_2\)) 3.33 (1H, br s, OH), 2.48 (3H, s, CH\(_3\)), 1.90 (s, CH\(_3\), second isomer?, 23%) and 1.23 (3H, t, \(J 7\), CH\(_3\)); \(\nu\) max. 3300 (br, OH), 2900 (CH str), 1725 (ester CO), 1700 (CO), 1630 (C=N), 1375 (CH bend) and 1240 (C-O-C).

**D/H Exchange in ethyl \(\alpha\)-nitrosoacetoacetate.**— When deuterium oxide was added to ethyl \(\alpha\)-nitrosoacetoacetate in DMSO, the hydroxyl peak (\(\delta 3.33\)) in the p.m.r. spectrum disappeared. After storing at
35° for 3 days, the intensity of the methyl group next to the carbonyl group was decreased by 22% relative to the intensity of the methyl group in -CH₂CH₃.

**t-Butyl α-nitrosoacetoacetate.** This ester was prepared as the abovementioned ethyl compound, using t-butyl acetoacetate (45 g) and sodium nitrite (22 g; 1.74 mol.equiv.). The work-up was slightly different from the above: before extraction of the orange-yellow emulsion with ether the following day, another aliquot of water (184 ml) was added. The combined ether extracts were further washed once with saturated aqueous urea. The yield was 53.3 g, crude; δ(CCl₄) 2.30 (3H, s, CH₃), 2.07 (1H, s, OH) and 1.50 (9H, s, 3 x CH₃); νmax. 3300 (br, OH), 3000-2900 (CH str), 1725 (ester CO), 1700 (CO), 1635 (C=N), 1375 (CH bend) and 1260 (C-O-C).

**D/H Exchange in t-butyl α-nitrosoacetoacetate.** On addition of deuterium oxide to t-butyl α-nitrosoacetoacetate in CCl₄, the hydroxyl peak (δ 2.07) in the p.m.r. spectrum disappeared, but the other protons were not exchanged.

**Hydroxyiminomalononitrile.** Nitrosation of malononitrile in acetic acid was carried out following the method of Ferris, Sanchez and Mancuso. The final solution of hydroxyiminomalononitrile (in a 1:1 mixture of tetrahydrofuran and ether) was used directly in the aminomalononitrile synthesis.

**Purification of hydroxyiminomalononitrile via its silver salt.** Malononitrile (5 g) was nitrosated as above and a solution of silver nitrate (15 g; 1.1 mol.equiv.) in water (15 ml) was added dropwise
to the solution of the formed oxime. The orange-yellow precipitate was collected, washed with water and dried to give hydroxyimino-malononitrile silver salt (15.5 g; 100%); $\nu_{\text{max.}}$ 2240 (CN), 1330, 1245 and 1235. The salt was dissolved in 8N-nitric acid (35 ml), and the free oxime was extracted into ether (100 ml) and was used directly in the following step (synthesis h; see Table 2.4, p.101).

Aminomalonic nitrile p-toluenesulphonate was prepared by reducing the preceding hydroxyimonomalononitrile with aluminium amalgam. After filtration, the amino compound was precipitated with p-toluene-sulphonic acid monohydrate. For yield and m.p., see Table 2.4 [lit.\(^{135}\) yield 78-82%; m.p. 172\(^\circ\) (from acetonitrile)]; $\delta$(DMSO) 7.32 (4H, q, $C_6H_4$), 5.97 (1H, br s, CH) and 2.23 (3H, s, $C_6H_4$-CH$_3$); $\nu_{\text{max.}}$ 3450 (br, NH), 2920-2520 (several peaks, CH str); 2140 (vw, b, CN), 1610 (NH bend), 1190, 1060 and 690 (RSO$_3$), 1130-1160, 1035, 1010 and 820 (C$_6$H$_4$).

In spite of following Ferris, Sanchez and Mancuso's detailed description of the method in 'Organic Syntheses' in every respect, the synthesis was quite often irreproducible. In a dozen attempts (see Table 2.4) the following difficulties were encountered: the aluminium amalgam reduction was difficult to initiate or would not start at all, even though the mixture was boiled. When it did proceed, stirring soon stopped because aluminium amalgam and aluminium hydroxide became embedded in an apparently polymeric gum which was difficult to break up. In this case, a light grey-red compound could be isolated from the solvent which had a melting point above 360\(^\circ\). In the abortive synthesis b (see Table 2.4) the following peaks were missing in the i.r. spectrum of the product:
2920-2520 (vw), 2140 (CN) and 1620 (NH bend); $\delta$(DMSO) 7.32
(4H, q, C$_6$H$_4$), 3.40 (1H, br s, ?) and 2.23 (3H, s, C$_6$H$_4$-CH$_3$).

In synthesis c, the i.r. spectrum of the polymeric material was
almost identical with that of the aminomalononitrile salt, but the
synthesis was nevertheless unsuccessful because the product did not
yield the pyrazine; $\delta$(DMSO) 7.30, 5.00 (2.5H, br s, ?) and 2.23.
Although polymeric, its solubility was as high as $\approx$400 mg in 1 ml
of water. Another disadvantage was that the p-toluenesulphonic
acid, if added too rapidly, seemed to form an oily phase consisting
of 'melted' acid. The salt formed tended to dissolve in the
latter phase, and crystallization was difficult.

Improvements for the preparation of aminomalononitrile
p-toluenesulphonate.

In order to succeed in this synthesis, it is necessary to add
the following notes to the prescribed method. (i) To make the
aluminium amalgam as reactive as possible. This was done according
to the method of Wislicenus and Kaufmann. The aluminium foil was
rinsed thoroughly with ether, dried and cut into half-inch squares.
These were etched with 2N-sodium hydroxide to a point of strong
hydrogen evolution, the solution was decanted, and the aluminium was
washed once superficially with water so that it retained some alkali.
It was then treated with a 0.5% mercuric chloride solution for 1-2 min,
and the entire process was repeated twice. The shiny amalgamated metal
was washed twice with water, once with ethanol and twice with tetra-
hydrofuran before use. (ii) It was found that the main reason why the
reaction did not start was lack of a sufficient amount of water which
was necessary for the reduction (see Equation b in Scheme 2.3, p.51).
The water content in water-saturated-ether was far below
the required amount for the reaction, and it had to be added very slowly to the mixture at room temperature, or slightly above it, in order to initiate the reaction. When the reaction became exothermic, external cooling with a dry ice-acetone bath was necessary. The water (a total of 1 ml per g of malononitrile) was added at such intervals as to keep the exothermic reaction going. It was, however, experienced that the reduction could still fail, even after addition of water. (iii) Because the reaction becomes vigorous, it is advisable to use as large a reaction flask as possible (preferably 5 litres for a 25 g scale), and at the same time to have a dry ice-acetone bath ready for immediate cooling; (iv) In case there is no significant heat evolution or the aluminium amalgam remains mainly unreacted even after heating, it is unnecessary to work the mixture up. The reduction has not, for some as yet unexplained reason, taken place. The whole reaction will then have to be repeated. (v) The time lapse between reduction and precipitation with p-toluenesulphonic acid should be as short as possible to avoid decomposition of the aminomalsononitrile formed. This was done by shortening, or completely eliminating, the time during which the mixture was boiled under reflux. As soon as most of the amalgam was oxidized (when the exothermic reaction subsided), the mixture was cooled in ice. The aluminium salts were removed by centrifugation instead of filtration. (vi) Before use, the p-toluenesulphonic acid monohydrate was dehydrated by heating on a steam bath for 4 h under water-pump vacuum.
Table 2.4

Data from aminomalononitrile $p$-toluenesulphonate and pyrazine

1-oxide syntheses

<table>
<thead>
<tr>
<th>Amount $^a$</th>
<th>Yield $^b$ (%)</th>
<th>Yield $^γ$ (%)</th>
<th>M.p. °C</th>
<th>% pyrazine 1-oxide $^δ$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) 0.096</td>
<td>49</td>
<td>0</td>
<td>177-178.5</td>
<td>80</td>
</tr>
<tr>
<td>(b) 0.38</td>
<td>0</td>
<td>13</td>
<td>&gt;360</td>
<td>0</td>
</tr>
<tr>
<td>(c) 0.38</td>
<td>0</td>
<td>69</td>
<td>&gt;360</td>
<td>0</td>
</tr>
<tr>
<td>(d) 0.38$^ε$</td>
<td>0</td>
<td>45</td>
<td>&gt;360</td>
<td>0</td>
</tr>
<tr>
<td>(e) 0.38$^ε$</td>
<td>55 (brown</td>
<td>-</td>
<td>-</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>oil)</td>
<td>17$^ε$ (oil)</td>
<td>-</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>2$^ε$</td>
<td>169-170</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td>(f) 0.38</td>
<td>0</td>
<td>59</td>
<td>&gt;360</td>
<td>0</td>
</tr>
<tr>
<td>(g) 0.19</td>
<td>7</td>
<td>-</td>
<td>-</td>
<td>56</td>
</tr>
<tr>
<td>(h) 0.074</td>
<td>14$^ζ$</td>
<td>170-171.5</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>(i) 0.38$^ε$</td>
<td>65</td>
<td>58, 7</td>
<td>170-170.5</td>
<td>98</td>
</tr>
<tr>
<td>(j) 0.76</td>
<td>0</td>
<td>47</td>
<td>&gt;360</td>
<td>0</td>
</tr>
<tr>
<td>(k) 0.76$^ε$</td>
<td>42</td>
<td>0</td>
<td>170-170.5</td>
<td>87</td>
</tr>
<tr>
<td>(l) 0.38</td>
<td>68</td>
<td>0</td>
<td>170-171</td>
<td>91</td>
</tr>
</tbody>
</table>

(a) moles of malononitrile

(b) yield of aminomalononitrile $p$-toluenesulphonate

(γ) yield of unidentified polymeric compound

(δ) yield of pyrazine 1-oxide formed when product was condensed with hydroxyiminoacetone

(e) recrystallized from acetonitrile

(ζ) hydroxyiminomalononitrile purified via silver salt.

Notes:
- M.p. = melting point
- Yield of unidentified polymeric compound is not stated for all entries.
- % pyrazine 1-oxide is stated for most entries.
- Some entries have multiple melting points or yields.
Stability of aminomalononitrile.— Aminomalononitrile p-toluenesulphonate (200 mg) was dissolved in water and basified with aqueous ammonia. Extraction with ether gave a light yellow oil which became dark brown on standing at 25° for 1 h. It was stored at 5° for a week and then extracted with ether (2 ml). The undissolved residue (~200 mg) consisted of black, glittering crystals which probably consisted of polymeric material; m.p. 360°; ν<sub>max</sub> 3360 (br, NH), 2220 (CN) and 1645 (br). No p-toluene-sulphonate salt could be precipitated from the ether extract.

D/H Exchange in aminomalononitrile p-toluenesulphonate.— P.m.r. studies showed that the C-hydrogen did not exchange when aminomalononitrile p-toluenesulphonate, dissolved in DMSO, was shaken with deuterium oxide.

Ethyl α-nitrosocyanoacetate.— This was prepared by nitrosation of ethyl cyanoacetate in the same way as ethyl acetoacetate (p. 96) in a good yield (80%); m.p. 130-131°. It was also prepared by a variation described by Conrad and Schulze, i.e. addition of acetic acid after having dissolved and mixed the ethyl cyanoacetate and sodium nitrite, which gave long prismatic crystals of ethyl α-nitrosocyanoacetate (83%); m.p. 132-133° (lit., yield 87%; m.p. 133°); δ(DMSO) 4.28 (2H, q, J 7, CH₂) and 1.25 (3H, t, J 7, CH₃); ν<sub>max</sub> 3220, 3140 (s, OH, NH), 3000-2800 (CH str), 2240 (w, CN), 1725 (ester CO), 1440, 1320 and 1075.

Ethyl α-aminocyanoacetate p-toluenesulphonate.— The preceeding nitroso compound was reduced with aluminium amalgam and, after filtration, the amino compound formed was precipitated with
anhydrous p-toluenesulphonic acid as for the synthesis of aminomalononitrile p-toluenesulphonate\(^\text{158}\) (p. 98) to give ethyl \(\alpha\)-aminocyanoacetate p-toluenesulphonate (54\%); m.p. 129-130\(^\circ\)C (lit.\(^\text{158}\), yield 34\%; m.p. 115-117\(^\circ\)C); \(\delta(DMSO)\) 7.30 (4H, q, \(C_6H_4\)), 5.90 (\(\gamma\)3H, s, \(NH^+\)), 4.27 (2H, \(J\) 7, \(CH_2CH_3\)), 2.26 (3H, s, \(C_6H_4-CH_2\)) and 1.23 (3H, \(J\) 7, \(CH_2CH_3\)); \(\nu_{\text{max.}}\) 3460 (NH str), 2950 (CH str), 2130 (w, CN), 1770 (ester CO), 1320, 1260, 1245, 1125, 1040 and 1015.

A number of unsuccessful attempts at reducing ethyl \(\alpha\)-nitrosocyanoacetate was carried out at the same time when attempts were made at reducing the hydroxyiminomalononitrile. In the following, a short summary is given of these abortive attempts.

Ohta's method,\(^\text{159}\) i.e. addition of aluminium amalgam to the solution of nitroso compound at a temperature below 25\(^\circ\)C, failed. Addition of a few small lumps of 10\% sodium amalgam did not initiate the reduction. Wilson reported that the Al/Hg reduction was unsuccessful and recommended reduction with zinc dust in glacial acetic acid. However, this procedure did not yield the amino compound in this work, even after increasing both the reaction time and the amount of zinc. The experiments were monitored by i.r. spectroscopy. P.m.r. spectra showed that a zinc complex was not formed. Adjusting the pH of the reaction mixture to \(\gamma\)9 before extraction with chloroform and later ether also proved unsuccessful. Hydrogenation over 10\% Pd/C in the presence of \(p\)-toluenesulphonic acid, or Raney nickel similarly failed to reduce the nitroso compound successfully.
D/H Exchange in ethyl α-aminocynoacetate p-toluenesulphonate.—
P.m.r. studies showed that the C-hydrogens did not exchange when ethyl α-aminocyanoacetate p-toluenesulphonate, dissolved in DMSO, was shaken with deuterium oxide.

2-Amino-3-cyano-5-methylpyrazine 1-oxide.— A suspension of hydroxyiminoacetone (1.74 g) and aminomalononitrile p-toluenesulphonate (5.06 g; 1.0 mol.equiv.) in propan-2-ol (20 ml) was stirred at room temperature for 4 h. Taylor claimed that the product crystallized directly from the reaction mixture as yellow crystals. This was, however, difficult to observe because the suspension was heterogeneous throughout the reaction. The pyrazine 1-oxide (2.39 g; 80%; lit.132 83%) was collected, washed with cold water followed by cold ethanol and dried. Recrystallization (30%) from ethanol did not increase the m.p. (189-190°; lit.132 187-188°); the yield varied greatly with the quality of the aminomalononitrile p-toluenesulphonate that was used for the condensation (see Table 2.4, p.101); δ(6N-DC1) 8.73 (1H, s, H(6)) and 2.57 (3H, s, CH₃(5)); δ(CDC1₃) 8.08 and 2.42 (lit.,132 δ(CD₃COOD) 8.03 and 2.00) ν max. 3400-3200 (NH str), 2255 (CN), 1645 (NH bend) and 1220 or 1170 (N⁺O⁻); the u.v. spectrum (in EtOH) was identical with the literature record;132 the u.v. spectra of the pyrazine 1-oxide in buffers, pH 2, 5 and 9, were all identical.

D/H Exchange in 2-amino-3-cyano-5-methylpyrazine 1-oxide.—
The pyrazine 1-oxide (40 mg) was dissolved in 6N-DC1 (0.5 ml) and stored at 35° in a sealed n.m.r. tube. The relative intensities of the peaks in the p.m.r. spectrum were measured, C(6)/CH₃
(time in days): 1.0/3.0 (0), 0.8/2.7 (1), 0.7/2.6 (2) and 0.2/0.0 (10). The i.r. spectrum of the pyrazine 1-oxide resembled that of the hydrochloride. The i.r. spectrum of the deuterated pyrazine 1-oxide, however, was slightly different from that of the non-deuterated compound.

2-Amino-3-cyano-6-deutero-5-trideuteromethylpyrazine 1-oxide.— Perdeuterated hydroxyiminoacetone (5.8 g) and aminomalonalonitrile p-toluenesulphonate (16.9 g; 1.05 mol.equiv.) in propan-2-ol (100 ml) were stirred at 25° for 4 h. The pyrazine oxide that separated contained some p-toluenesulphonic acid which was removed by dissolving the mixture in water, adjusting the pH to 9 and extracting with chloroform. Evaporation gave the deuterated pyrazine oxide (7.26 g; 74%) which had m.p. 188.5–189°, after sublimation at 125° and 0.1 mmHg (lit. 187–188° for non-deuterated oxide); the H(6) signal was absent in the p.m.r. spectrum, and the methyl signal (δ ~2.57) was very weak and slightly broadened because of geminal H-D coupling; the u.v. spectrum was identical with that of the non-deuterated oxide; ν (max. 3405, 3310 (NH), 2150 (CN), 1645 and 1625; t.l.c. gave only one spot (Rf = 0.85; silica, eluant A, see p. 119); m/z 156 (2%), 155 (13%), 154 (100%, C₆H₂D₄N₄O⁺), 153 (39%, C₆H₃D₃N₄O⁺), 152 (5%, C₆H₄D₂N₄O⁺), 151 (1%) and 150 (0) (Found: C, 46.7; H+D, 6.3; N, 36.2. C₆H₂D₄N₄O requires C, 46.75; H+D, 6.5; N, 36.3%).

2-Amino-3-cyano-5-methylpyrazine was prepared by deoxygenation of the corresponding 1-oxide by (a) phosphorus trichloride and (b) ferrous sulphate:
(a) A general procedure for reduction of pyrazine 1-oxide to the corresponding pyrazines was described by Taylor and coworkers.\textsuperscript{132} 2-Amino-3-cyano-5-methylpyrazine 1-oxide (9.12 g) was dissolved in dry tetrahydrofuran (400 ml). The solution was cooled to 0\textdegree C and treated with PCl\textsubscript{3} (47.2 g; 5.65 mol equiv.; lit\textsuperscript{132} 23.4 mol equiv.) over a period of 10 min. The reaction mixture was stirred at room temperature for 40 min, concentrated to ca. 50 ml and poured into ice-water (1 litre). Taylor et al. do not report any difficulties at this stage, but were able to collect a precipitate. It was, however, often experienced that precipitation did not occur and was difficult to initiate. The reaction mixture had to be concentrated until turbidity whereupon crystallization could be induced at 0\textdegree C. The solid was collected, washed well with water and dried overnight in a desiccator over KOH. The yellow pyrazine (5.41 g; 61\%) had m.p. 169-170\textdegree C; 171-172\textdegree C after sublimation at \nabla 150\textdegree C and 0.1 mmHg (lit\textsuperscript{132}; m.p. 172-173\textdegree C); the p.m.r. spectrum and the u.v. spectra (identical in buffers of pH 2, 7, 12 and in methanol) corresponded with those reported in the literature\textsuperscript{132} (u.v. in methanol); \nu_{max.} 3450, 3360 (NH str), 2245 (CN), 1660, 1640, 1305 and 1195.

(b) Deoxygenation of 2-amino-3-cyano-5-methylpyrazine 1-oxide by ferrous sulphate.—To a solution of 2-amino-3-cyano-5-methylpyrazine 1-oxide (1.50 g) in water (150 ml) was added ferrous sulphate (FeSO\textsubscript{4}, 7 H\textsubscript{2}O; 6.00 g; 1.08 mol equiv.). 11N-Ammonia (10 ml) was added and the mixture was boiled under reflux for 3 h. The black precipitate of hydroxide (no u.v. spectrum) was removed by filtration. The filtrate was concentrated to half its volume which caused the pyrazine (660 mg; 49\%) to crystallize. The u.v. spectra at pH 2, 7, 12, and in methanol were all identical and corresponded with the literature values [\lambda_{max.} MeOH, 249 (4.17) and 358 (3.88)].
2-Amino-3-cyano-6-deutero-5-trideuteromethyl (partial) pyrazine. – This was prepared, in a 46% yield, by deoxygenation of the corresponding 1-oxide with PCl₃ as described for the non-deuterated compound. After addition of water it was necessary to concentrate the reaction mixture to turbidity, and to cool. The deuterated pyrazine had m.p. 174.5-175.5° after sublimation; the u.v. spectra were identical with those of the non-deuterated pyrazine; \( \lambda_{\text{max}} \) 2140 (CN) and 1215 (s) (Found: C, 53.1; H+D, 5.6; N, 41.3. \( \text{C}_6\text{H}_4\text{D}_1\text{.5N}_4 \) requires C, 53.1; H+D, 5.6; N, 41.3%); and the H(6) signal was absent in the p.m.r. spectrum.

Attempts to prepare 2-amino-3-cyano-6-ethoxycarbonyl-5-methylpyrazine 1-oxide by condensing ethyl \( \alpha \)-nitrosoacetoacetate (1.87 g; 1.4 mol.equiv.) with aminomalononitrile \( p \)-toluenesulphonate (2.53 g) in propan-2-ol (15 ml) by stirring for 4 and 7 days were unsuccessful, and all the starting material was recovered.

2-Amino-3-carbamoyl-5-methylpyrazine 1-oxide. – 2-Amino-3-cyano-5-methylpyrazine 1-oxide (200 mg) was dissolved in sulphuric acid (\( d \) 1.8, 1 ml) (%25 mg of pyrazine formed a stonehard lump which was removed). At different times (t), aliquots were collected, diluted with water (0.5 ml) and neutralized with \( 11\text{N} \)-ammonia to pH ≥7. The light yellow amide precipitated. Chromatography on Whatman No. 4 paper, using 3% NH₄Cl, gave the following ratios, nitrile/amide (time in min): 1/2 (15) and 1/10 (120). When hydrolysis was carried out on a steam bath, the conversion was completed after 10 min and yielded 192 mg (85%) of amide; m.p. 228-230°. In a third attempt, the cyanopyrazine (5.0 g) in sulphuric acid (\( d \) 1.8, 25 ml) was heated at 100° for 15 min, poured into water and neutralized with aqueous ammonia. The yellow solid was collected and sublimed at
180-190°/0.1 mmHg to give the amide (84%); m.p. 230-230.5° (lit. 131 m.p. 218-219° and 161 m.p. 235-236°, prepared from α-aminocyanacetamide and hydroxyiminoacetone); ν max. 3470, 3440 and 3400 (NH), 1695, 1670 (amide), 1620 (C=Ν) and 1170 (Ν-O) (Found: C, 43.1; H, 4.8; N, 33.5. Calc. for C₆H₆N₄O₂: C, 42.8; H, 4.8; N, 33.3%). The amide could not be hydrolysed to the acid by prolonged heating in sulphuric acid.

2-Amino-3-carbamoyl-5-methylpyrazine.— To a solution of 2-amino-3-carbamoyl-5-methylpyrazine 1-oxide (504 mg) in boiling water (15 ml) was added sodium dithionite (6.0 g) portionwise. The mixture was boiled under reflux for 2 h, the resulting suspension was cooled and the precipitate was collected, washed with water and dried to give 324 mg (71%) of light yellow crystals; m.p. 187-192°.

The pyrazine was purified by sublimation at ~200°/0.1 mmHg; m.p. 201-202° (lit. 131 m.p. 203-204°); δ(CF₃COOH) 7.87 (1, s, C(6)-H) and 2.53 (3, s, C(5)-CH₃) (Found, after drying for 6 h at 80°/0.1 mmHg): C, 47.3; H, 5.2; N, 36.6. Calc. for C₆H₆N₄O: C, 47.4; H, 5.3; N, 36.8%).

2-Amino-3-carbamoyl-5-methylpyrazine from partial catalytic reduction of 2-amino-3-carbamoyl-5-methylpyrazine 1-oxide.— The pyrazine 1-oxide (504 mg) was dissolved in absolute ethanol (25 ml) and the catalyst, 5% Pd/C (504 mg), was added. After shaking with hydrogen at 20° and 720 mmHg for 90 min, the theoretical amount of hydrogen had not been absorbed, so 10% Pd/C (250 mg) was added and hydrogenation continued. The uptake of hydrogen stopped when a total of 80 ml (1.4 mol.equiv.) had been absorbed (t = 150 min).
The catalyst was removed and the filtrate freeze-dried to give 390 mg (86%) of light yellow solid; m.p. 179-180°C (sublimation did not increase the melting point; lit.131 m.p. 203-204°C). The i.r. spectrum showed that the product was a mixture of pyrazine and pyrazine 1-oxide. The microanalysis suggested 48% 1-oxide (Found, after drying for 6 h at 80°C/0.1 mmHg: C, 45.5; H, 5.2; N, 34.9. Calc. for 48% C₆H₈N₄O₂ + 52% C₆H₈N₄O: C, 45.2; H, 5.1; N, 35.1%).

In an abortive attempt to prepare 2-amino-3-methoxycarbonyl-5-methylpyrazine 1-oxide from 2-amino-3-cyano-5-methylpyrazine 1-oxide, the latter compound (300 mg) was treated with methanolic HCl (10 ml) on a steam bath. Even after boiling under reflux for 5 h, i.r. spectroscopy indicated that the nitrile was unreacted. Also, sulphuric acid (method of Rydon and coworkers162) instead of hydrogen chloride, in methanol did not react with the nitrile.

2-Amino-3-ethoxycarbonyl-5-methylpyrazine 1-oxide was prepared by the method of Taylor and coworkers.131 A solution of hydroxy-iminoacetone (0.87 g) and ethyl α-aminocyanacetate p-toluene-sulphonate (3.0 g; 1.0 mol.equiv.) in absolute methanol (5 ml) was stirred for 24 h at 25°C (lit131 35°C). The condensation [followed by u.v. spectroscopy (t½ = ~3 h)] gave a yield of 950 mg (48%; lit131 49-58%) and attempts to improve the yield by adjusting the pH of the mother liquor to ~7 with N-NaOH followed by extraction with chloroform (6 x 25 ml) were unsuccessful; m.p. 131-132°C (lit., m.p. 132-133.5°C); δ(CDC13) 8.22 (1H, s, δ(6)-H), 4.5 (2H, q, δ 7, CH₂CH₃), 2.47 (3H, s, δ(5)-CH₃) and 1.43 (3H, t, δ 7, CH₂CH₃) (lit.131, 8.13, 4.5, 2.43 and 1.40); ν max. 3470, 3350 (NH str), 3000-2900 (CH str), 1690 (ester CO), 1615, 1550-1540, 1410, 1350
and 1175 (N\(^{+}O^{-}\)); \(\lambda_{\text{max.}}^{\text{PH 2}} = \lambda_{\text{max.}}^{\text{PH 7}} = \lambda_{\text{lit.}}^{\text{(H\(_2\)O)}^{131}}(\log \varepsilon), 226 [4.05], 246 [4.29] \) and 378 [3.94]; \(\lambda_{\text{max.}}^{\text{PH 12}} = 225\text{sh} (0.52), 239 (1.00) \) and 364 (0.43); \(R_f 0.75 \) (Whatman No.4 paper, 3% NH\(_4\)Cl).

2-Amino-6-deutero(partial)-3-ethoxycarbonyl-
5-trideuteromethyl(partial)pyrazine.—2-Amino-6-deutero-3-ethoxycarbonyl-5-trideuteromethyl(partial)pyrazine 1-oxide (3.0 g) was dissolved in absolute ethanol (300 ml) containing 5% Pd/C (3 g). The mixture was shaken with hydrogen at 20°C and 720 mmHg. After the theoretical absorption of hydrogen (90 min) the catalyst was filtered off, and the filtrate evaporated. The residue (92%) was sublimed at \(\nu 70^0/0.5 \text{ mmHg} \) to give the pure pyrazine (78%) which had m.p. 85-87°C; \(\delta(CDCl\text{)}_3 8.20 \) (s, \(\varepsilon(6)-H, 20\% D), 4.5 \) (2H, q, \(J 7, CH\text{CH}_3\)), 2.52 (s, \(\varepsilon(5)-CH\text{3}, 22\% D) \) and 1.45 (3H, t, \(CH\text{CH}_3\)). P.m.r. spectra of four preparations of this deuterated pyrazine showed that 0-85% of the deuterium on \(\varepsilon(6)\) and 0-20% of the deuterium on the partially deuterated \(\varepsilon(5)\)-methyl group was lost during the catalytic reduction.

\(v_{\text{max.}} 3465, 3310 (\text{NH}), 1690 (\text{ester CO}), 1615 (\text{NH}), 1380 (\text{CH bend}), 1320 \) but no \((N^{+}O^{-})\) peak; \(\lambda_{\text{max.}}^{\text{PH 2}} = \lambda_{\text{max.}}^{\text{PH 12}} = 250 (1.00) \) and 361 (0.60) (Found, after drying at 50°C for 12 h: C, 52.7; H+D, 6.7; N, 23.4. \(C_8H_{10}DNO_2\) requires C, 52.7; H+D, 6.6; N, 23.1%).
2-Amino-6-deutero-3-ethoxycarbonyl-5-trideuteromethyl-
(partial)pyrazine 1-oxide.—Perdeuterated hydroxyiminoacetone
(2.2 g) and ethyl α-aminocyanacetate p-toluenesulphonate
(7.6 g; 1.05 mol. equiv.) in methanol (10 ml) was stirred at
35° for 24 h. The solution was evaporated, diluted with water
(40 ml), the pH adjusted to 9, and the mixture extracted with
chloroform. Evaporation of the extract gave the deuterated
pyrazine oxide (3.3 g; 69%) which had m.p. 132-133.5° after
sublimation at 140° and 0.5 mmHg; m/z 185 (28%, C$_8$H$_7$D$_4$N$_3$O$_2$•$^+$),
184 (71%, C$_8$H$_8$D$_3$N$_3$O$_2$•$^+$), 183 (100%, C$_8$H$_9$D$_2$N$_3$O$_2$•$^+$), 182 (85%,
C$_8$H$_{10}$D$_{N}$_3O$_2$•$^+$) and 181 (0); ν$_{max}$ 3470, 3335 (NH), 1690 (CO),
1610 and 1520; the u.v. spectrum was identical with the reported
spectrum$^{131}$ of the non-deuterated oxide; δ(CDC$_1$$_3$) 1.45
(3, t, J 7, CH$_3$), 2.47 (2, s, ξ(5)-CH$_3$), 4.51 (2, q, J 7, CH$_2$)
and 7.3 (s, br s, NH$_2$) with no signal for ξ(6)-H (Found:
C, 48.3 H+D, 6.7; N, 21.1. C$_8$H$_9$D$_2$N$_3$O$_3$ requires C, 48.2;
H+D, 6.6; N, 21.1%).

2,4-Diamino-6-methylpteridine 8-oxide was prepared by the
method of Taylor and coworkers$^{132}$ which gave a yield of 76% with
m.p. >360° (lit.$^{132}$ 81%, m.p. >320°); δ(CF$_3$COOH) 8.73 (1H, s, ξ(7)-H)
and 2.75 (3H, s, ξ(6)-CH$_3$); the chemical shifts reported in the
literature (8.43 and 2.25)$^{132}$ are slightly different. However,
the p.m.r. spectrum of the 8-oxide obtained from a second prepara-
tion was identical with the values previously recorded in this
work; ν$_{max}$ 3400, 3330 (NH str), 1630 (NH bend) and 1175 (N⁺-O⁻);
the u.v. data were identical with the literature values$^{132}$(Found:
C, 43.5; H, 4.3; N, 43.4. Calc. for C$_7$H$_6$N$_6$O: C, 43.7; H, 4.2;
N, 43.7%).
2,4-Diamino-6-methylpteridine was prepared in the same way as the corresponding 8-oxide, using 2-amino-3-cyano-5-methylpyrazine (5.0 g), guanidine hydrochloride (4.1 g; 1.2 mol. equiv.) and sodium (2.6 g; 3.0 mol. equiv.) in absolute methanol (350 ml) which gave a yield of 4.75 g (73%) with m.p. >360° (lit.\(^{132}\), yield 84%, m.p. >340°); \(\delta(CF_3COOH)\) 8.33 (1H, s, \(\zeta(7)\)-H) and 2.78 (3H, s, \(\zeta(6)\)-CH\(_3\)) (lit.\(^{132}\), 8.16 and 2.30); \(\nu_{\text{max.}}\) 3480, 3330 (NH str), 1665, 1640, 1625 and 1445; the u.v. spectrum (in pH 2 buffer) was identical with the one in the literature; \(\lambda_{\text{max.}}^{\text{pH} 7} = \lambda_{\text{max.}}^{12} 255(1.00)\) and 371 (0.43) (Found, after drying at 100°/0.5 mmHg for 4 h: C, 47.3; H, 4.8; N, 47.4. Calc. for C\(_7\)H\(_8\)N\(_6\): C, 47.7; H, 4.6; N, 47.7%).

D/H Exchange in 2,4-diamino-6-methylpteridine.— The pteridine (100 mg) was dissolved in 2N-NaOD (15 ml) at 100° and heated on a steam bath in a sealed tube overnight. The yellow solid that precipitated on cooling (56 mg) was collected, washed with water followed by ethanol, and dried. The product had m.p. >360° (decomp.) and had t.l.c. properties and u.v. spectra identical with those of an authentic sample of 6-methylpterin. The p.m.r. spectrum in D\(_2\)SO\(_4\) showed no signal from the \(\zeta(6)\)-methyl group, whereas hardly any exchange of H(7) had occurred indicating that the product was 6-trideuteromethylpterin (sodium salt) (Found, after drying at 100° for 6 h: C, 39.9; H+D, 4.9; N, 33.0; Na, 10.9. C\(_7\)H\(_3\)D\(_3\)N\(_0\)Na, 0.5 H\(_2\)O requires C, 39.8; H+D, 4.8; N, 33.2; Na, 10.9%).
2,4-Di amino-7-deutero-6-trideuteromethyl(partial)pteridine.
Deuterated 2-amino-3-cyano-6-deutero-5-trideuteromethyl(partial)-
pyrazine (D/H = 2/4; 5.0 g) was added to a methanolic solution of
guanidine [from guanidine hydrochloride (4.1 g; 1.2 mol. equiv.)
dissolved in methanol (350 ml) containing sodium (2.6 g; 3.1 mol.
equiv.)], and the mixture was stirred at room temperature. As no
solid had separated after 16 h, the solution was boiled under reflux
for another 18 h. The yellow solid that separated on cooling
(4.7 g; 71%) was collected, washed with water, dried and recrystall-
ized from N,N-dimethylformamide in 46% yield. The pteridine had
m.p. >360° (decomp.), and its t.l.c. properties and u.v. spectra
were identical with an authentic sample of non-deuterated diamino-
pteridine. The p.m.r. spectrum in 2N-deuterium chloride showed no
peak for $\xi(7)$-H, and the $\xi(6)$-methyl signal was weak and broad
because of geminal H-D coupling (Found: C, 46.7; H+D, 5.6;
N, 46.4. C$_7$H$_6$D$_2$N$_6$, 0.15 H$_2$O requires: C, 46.5; H+D, 5.7;
N, 46.5%).

Reduction of 2,4-diamino-6-methylpteridine 8-oxide.
A. Using palladium on charcoal.—The pteridine 8-oxide (1.92 g)
was dissolved in absolute methanol, and the catalyst, 10% Pd/C
(960 mg), was added. The mixture was shaken with hydrogen at
20°/~720 mmHg. When the absorption had stopped (15 h), the
catalyst was removed and the solution freeze-dried which left a
light yellow compound (1.53 g; 86%, based on mw for dihydro
compound); m.p. >320°. The p.m.r. and i.r. spectrum showed that
the product was 2,4-diamino-6-methyl-7,8-dihydropteridine;
$\delta$(CF$_3$COOH) 4.36 (2H, s, $\xi(7)$-CH$_2$) and 3.00 (3H, s, $\xi(6)$-CH$_3$)
Attempts to prepare this 7,8-dihydro compound by reduction of the 8-oxide with sodium dithionite as described by Taylor and coworkers \(^{132}\) were not uniformly successful.

B. Using pre-reduced platinum oxide— The pteridine 8-oxide (1.9 g) was dissolved in absolute methanol containing a little dry HCl. The solution was added to a pre-reduced suspension of platinum oxide (192 mg) in methanol and shaken with hydrogen as above. After shaking for 18 h the theoretical amount of hydrogen had not been consumed, so a fresh portion of pre-reduced catalyst (192 mg) was added together with saturated methanolic HCl (40 ml). After a total of 20 h shaking, 3 mol.equiv. of hydrogen had been absorbed. The mixture was filtered and freeze-dried which left an almost white solid (1.95 g; 77% based on mw 253.1 (see analysis)) m.p. >230° (decomp.). The compound (200 mg) was recrystallized from 0.1N-HCl (15 ml) in a 47% yield. The resulting purple colour of the solid showed that some reoxidation had occurred. The spectroscopic data were identical with those obtained from an authentic sample of 2,4-diamino-6-methyl-5,6,7,8-tetrahydropteridine hydrochloride (see below).

2,4-Diamino-6-methyl-5,6,7,8-tetrahydropteridine hydrochloride.— 2,4-Diamino-6-methylpteridine \(^{132}\) (2.5 g) was added to a pre-reduced suspension of platinum oxide (250 mg) in 3N-hydrochloric acid (250 ml) and shaken with hydrogen at 20° and 720 mmHg. After the theoretical absorption of hydrogen (3 h), the catalyst was filtered off, and the filtrate evaporated. The residue was recrystallized
from ethanol containing a little ethanolic hydrogen chloride and gave (quantitatively) the tetrahydropteridine hydrochloride which had m.p. >230° (decomp.) \( \lambda_{\text{max.}} \) (pH 2) 219 nm (log \( \varepsilon \) 4.21) and 274 nm (log \( \varepsilon \) 4.15) (Found, after drying at 100° and 0.5 mmHg for 6 h: C, 21.3; H, 5.0; Cl, 45.2. Calc. for C\(_7\)H\(_{12}\)N\(_6\) \( \cdot \) 5HCl, 1.75 H\(_2\)O: C, 21.3; H, 5.2; Cl, 45.0%). The HCl content decreased on further heating but the salt darkened in colour. P.m.r. spectrum is in Table 2.2, p. 75.

**General method for the preparation of 5,6,7,8-tetrahydropteridine hydrochlorides.**

The pteridine (150 mg) in trifluoroacetic acid (6 ml) was added to a pre-reduced suspension of platinum oxide (50-90 mg) in trifluoroacetic acid (4 ml) and shaken with hydrogen at 20°/\( \sim \)720 mmHg. When absorption was complete (2-5 h, as shown by the u.v. spectrum in 0.2N-HCl which consists of one band at 260-270 nm and no absorption above 285 nm) the catalyst was removed by filtration through a Pasteur pipette plugged with quartz wool, Celite and charcoal (\( \sim \)100 mg each), and the solution was forced with dry nitrogen under a slight pressure. The filtrate at 0° was treated with a solution of concentrated hydrochloric acid (0.2 ml) in methanol (8 ml), and diluted by slow addition of dry ether (60 ml), whereby the tetrahydropteridine hydrochloride separated in colourless microcrystals. After standing at 0° for 10 min the solid (70-95% yield) was collected by centrifugation, and washed free from trifluoroacetic acid with dry ether (3 x 30 ml) and collected by centrifugation; then it was dried at 100° in vacuo.

The salt was usually analytically pure at this stage but if necessary further purification was achieved by dissolving it in a solution of
hydrochloric acid in methanol as before, adding dry ether,
and collecting and washing the salt by centrifugation.

e-e- and trans-2,4-Diamino-7-deutero-6-trideuteromethyl-
(partial)-5,6,7,8-tetrahydropteridine hydrochloride was prepared as
the above from 2,4-diamino-7-deutero-6-trideuteromethyl(partial)-
pteridine (p.113) and had m.p. 238-240° (decomp.), and its u.v.
spectrum was identical with that of the non-deuterated pteridine
salt (Found: C, 27.7; H+D, 6.8; Cl, 27.1. C\textsubscript{7}H\textsubscript{10}D\textsubscript{2}N\textsubscript{6}, 2.3 HCl,
2 H\textsubscript{2}O requires: C, 27.8; H+D, 6.8; Cl, 27.0%), the p.m.r.
data are in Table 2.2, p.75.

6-Methylpterin 8-oxide was prepared by the method of Taylor
and coworkers.\textsuperscript{131} Yield 61%, m.p. >360° (lit:\textsuperscript{131} 70%, >320°), p.m.r.
and u.v. data were identical with the literature values\textsuperscript{131}; ν\textsubscript{\text{max.}}
3450 (NH str), 1705 (CO), 1665 (NH bend), 1535, 1490, 1335 and 1170
(N\textsuperscript{+}O\textsuperscript{-}) (Found, after drying at 100°/0.5 mmHg for 4 h: C, 43.7;
H, 3.8; N, 36.0. Calc. for C\textsubscript{7}H\textsubscript{7}N\textsubscript{5}O\textsubscript{2}: C, 43.5; H, 3.7; N, 36.3%).

Attempts to prepare 6-methylpterin 8-oxide from 2-amino-3-
ethoxycarbonyl-5-methylpyrazine 1-oxide by condensation with
(a) cyanamide or (b) S-methyl isothiouronium sulphate, or from
2-amino-3-carbamoyl-5-methylpyrazine 1-oxide with (c) guanidine in
the presence of methoxide, (d) cyanamide, (e) cyanogen bromide or
(f) ammonium thiocyanate, and attempts to prepare 6-methylpterin from
3-carbamoyl (g), or 3-ethoxycarbonyl (h), 2-amino-5-methylpyrazine by
condensation with guanidine in the presence of methoxide were
uniformly unsuccessful. The conditions for the reactions
involving the above mentioned reagents (a,b,c, etc.) were as
follows: (a) and (b) 3 mol. equiv., both heated on a steam
bath for 3 days; (c), (g) and (h) 1.3 mol. equiv. of guanidine,
as for preparation of the 2,4-diamino compound, (d) and (e)
3 mol.equiv., both fused together with the pyrazine on a steam bath
for 24 h, (f) 1.1 mol.equiv., in warm N-HCl (1.1 mol.equiv.),
heated on a steam bath for 1 h, stored at room temperature for
another h, evaporated to dryness over a period of 2-3 h, then
addition of water which was re-evaporated slowly; the dry residue
was finally heated on a steam bath for 4 h.

6-Methylpterin was prepared by the method of Semb.\textsuperscript{130}

To a solution of 2,4,5-triaminopyrimidin-6(1\textit{H})-one sulphate
(127.30 g) in boiling water (7 litres) was added sodium sulphite
(1500 g; 11.2 mol.equiv., Na\textsubscript{2}SO\textsubscript{3} \cdot 7 H\textsubscript{2}O). The clear solution,
just below the boiling point, was treated dropwise with an aqueous
solution of pyruvaldehyde (methylglyoxal; 250 ml; 15%; 1.02 mol.
equiv.) containing sodium pyrosulphate (95 g; 0.94 mol.equiv.).
A yellow solution started to crystallize out almost immediately,
and the mixture was allowed to cool to room temperature overnight.
The solid was collected by centrifugation, washed with water, and
ethanol and dried. The yield of the 6-methylpterin was quantitative.
The p.m.r. spectrum showed that the product consisted of 64% 6-isomer
[\(\delta(2N-DCI) 8.81 (1, s, \zeta(7)-H)\) and 2.80 (3, s, \(\zeta(6)-CH_3\))] and 36%
7-isomer [\(\delta(2N-DCI) 8.93 (1, s, \zeta(6)-H)\) and 2.77 (3, s, \(\zeta(7)-CH_3\))].
If addition of sodium pyrosulphate was omitted, a 1:1 mixture of the
6- and 7-methylpterin was obtained. If both sodium sulphite and
pyrosulphate were omitted, mainly the 7-substituted isomer (>95%)
was formed. The more soluble 2,4,5-triaminopyrimidin-6(1\textit{H})-one
chloride was sometimes used instead of the sulphate in order to
(a) reduce the amount of solvent, and (b) avoid catalyst poisoning
in later reduction.
6-Methylpterin sodium salt was obtained pure by recrystallization of the preceding mixture from 10 parts of 2N-sodium hydroxide (50% recovery) and had m.p. >360° (decomp.). \( \nu_{\text{max.}} \)
3470, 3390 (NH), 3190, 1655 (CO), 1630, 1590, 1560, 1535 and 1515;
\( \lambda_{\text{max.}} \) (pH 2 buffer) 215 (\( \varepsilon = 5.90 \times 10^3 \); 1.00), 245sh (0.75),
256sh (0.25) and 326 (0.54); (pH 7 buffer) 221 (0.93), 233 (0.92),
271 (1.00) and 349 (0.51); (pH 12 buffer) 251 (1.00), 273 sh (0.32)
and 365 (0.33) (Found, after drying at 150° for 12 h: C, 41.4;
H, 3.3; N, 34.4; Na, 11.3. Calc. for \( C_7 H_6 N_5 ONa \), 0.25 H\( _2 \)O:
C, 41.3; H, 3.2; N, 34.4; Na, 11.3%). The free base was prepared by acidifying an aqueous solution of the salt, and collecting and washing (H\( _2 \)O and EtOH) by centrifugation because conventional filtration was exceedingly slow. This compound was identical with a sample from Taylor's unequivocal synthesis.

**D/H Exchange in 6-methylpterin.**—6-Methylpterin (65 mg) was dissolved in 100° 2N-NaOD (5 ml) and stirred overnight at 100°. The pterin, which precipitated on cooling, was collected by centrifugation and washed well with ethanol. The p.m.r. spectrum revealed that the 6-methyl group had been extensively exchanged whereas H(7) had not been displaced by deuterium. Even after (a) further 24 h at 100°, or (b) 2 days in a sealed tube on a steam bath, and (c) another 2 days at 120° in a bomb, H(7) was not exchanged. The u.v. spectra and t.l.c. properties were identical with those obtained from authentic non-deuterated 6-methylpterin.

**6-Trideuteromethylpterin sodium salt.**—6-Methylpterin (1 g) in 2N-sodium deuteroxide (70 ml) was heated in a bomb at 100° for 24 h. The sodium salt (600 mg) that crystallized on cooling was
collected, washed with a little water and ethanol, and dried. It had m.p. >360° (decomp.) (Found: C, 39.7; H+D, 4.7; N, 32.9; Na, 10.7. \( \text{C}_7\text{H}_3\text{D}_3\text{N}_5\text{Na} \), 0.5 H\(_2\)O requires C, 39.8; H+D, 4.7; N, 33.2; Na, 10.9\%). Prolonged heating of the sodium deuteroxide solution at 120° did not cause H(7) to be displaced by deuterium.

Conversions of 2,4-diamino- to 2-amino-4-oxo-pteridines including their 8-oxides.

2,4-Diamino-6-methylpteridine could be converted to 6-methylpterin in boiling 48% hydrogen bromide for 30 min in 75% yield, and in boiling 12N-hydrogen chloride for 1 h (70% yield). These reaction conditions, however, did not cause complete conversion of 2,4-diamino-6-methylpteridine 8-oxide to the corresponding pterin 8-oxide. The latter reaction was achieved, in 90% yield, by boiling the pteridine 8-oxide in 2N-NaOH overnight.

When 2,4-diamino-6-methylpteridine 8-oxide was treated with sodium nitrite in 2N-HCl at 0°, only starting material was recovered. Treatment with N-NaOH at room temperature for 4 h followed by boiling for another 4 h proved partially successful (less than 10% pterin 8-oxide was formed) as did boiling with 3N-HCl for 6 h.

The conversions could be followed by t.l.c. on Merck precoated 'Silica Gel F\(_{254}\)' plates. A mixture of (A) butan-1-ol, butan-2-one, water and formic acid (6:6:1:0.3, by volume) and a mixture of (B) butan-1-ol, propan-2-one and 7.5N-ammonia (4:3:1, by volume) were used as eluants. The compounds were adsorbed so strongly by the silica that neither boiling methanol nor boiling trifluoroacetic acid could extract them.
7-Deutero-6-methylpterin sodium salt.—2,4-Diamino-7-deutero-
6-trideuteromethyl(partial)pteridine (200 mg, see below) in
2N-sodium hydroxide (75 ml) was stirred at 100° for 9 h. The
solution was concentrated until a solid crystallized, and was cooled.
The yellow sodium salt (120 mg) was collected as above. A further
60 mg of salt was obtained from the mother liquors. The u.v. spectra
and t.l.c. properties were identical with those of authentic non-
deuterated 6-methylpterin, and only a sharp ζ(6)-methyl signal was
present in the p.m.r. spectrum (Found: Na, 11.3. C\textsubscript{7}H\textsubscript{3}D\textsubscript{5}ONa
requires Na, 11.5%).

7-Deutero(partial)-6-methylpterin.—6-Methylpterin (1.5 g) was
suspended in deuterium oxide (100 ml) and reduced with dithionite as
described on the next page to give 7,8-dideuteropterin. This was
reoxidized to the 7-deutero(partial)pterin by permanganate as
described by Taylor et al.,\textsuperscript{131} and the whole procedure was repeated.
The final product (0.62 g; 41%) was shown by u.v. and p.m.r. to be
7-deutero(70%)-6-methylpterin (Found, after drying at 150°/0.5 mmHg
for 6 h: C, 50.7; H+D, 5.0; N, 41.8. C\textsubscript{7}H\textsubscript{6.3}D\textsubscript{0.7}O\textsubscript{0.7}N\textsubscript{5}, 0.25 H\textsubscript{2}O
requires C, 50.5; H+D, 5.0; N, 42.1%).

7-Deutero-6-trideuteromethylpterin sodium salt was prepared
as above from 2,4-diamino-7-deutero-6-trideuteromethyl(partial)-pt
eridine in 2N-sodium deuteroxide (18 h at 100°) and had m.p.
>360° (decomp.) (Found: C, 40.8; H+D, 5.2; N, 34.4.
C\textsubscript{7}H\textsubscript{1.5}D\textsubscript{4.5}N\textsubscript{5}Na, 0.1 H\textsubscript{2}O requires C, 40.9; H+D, 5.2; N, 34.1%).
The u.v. spectra and t.l.c. properties were identical with the above,
but it had no signals in the p.m.r. spectrum in 2N-deuterium
chloride.
6-Methyl-7,8-dihydropterin. 6-Methylpterin (1.5 g) was suspended in boiling water (150 ml). Sodium dithionite (18 g; 15 mol. equiv.) was added and the resulting reaction mixture stirred at room temperature overnight. The colourless precipitate was collected, washed with water followed by ethanol, and dried; yield 1.20 g (79%). The u.v. spectra (pH 2, 7 and 12) of the solid were identical with those of an authentic sample of 6-methyl-7,8-dihydropterin; \[ \text{mp. 300° (decomp.)} \]
\[ \delta(\text{CD}_3\text{COOH}) 5.08 (2H, s, \text{C}(7)-\text{CH}_2) \text{ and } 2.67 (3H, s, \text{C}(6)-\text{CH}_3); \nu_{\text{max.}} 3300 (\text{NH str}), 2900 (\text{CH str}), 1710 (\text{CO}), 1665 (\text{NH bend}) \text{ and } 1020 (\text{HSO}_3). \]
Taylor and coworkers prepared this compound in 90% yield by reducing the corresponding 8-oxide with dithionite.

6-Methyl-5,6,7,8-tetrahydropterin hydrochloride was prepared by catalytic reduction of 6-methylpterin following the general procedure. The tetrahydropterin had m.p. >260° (decomp.); the p.m.r. spectra (100 and 270 MHz) are in Figures 2.1 and 2.4. and in the Table 2.2, p. 75; \[ \nu_{\text{max.}} 3400 (\text{br, NH}), 2900 (\text{CH str}), 2705, 1720 (\text{CO}), 1690, 1665 \text{ and } 1575; \lambda_{\text{max.}} (\text{pH } 1) 266; (\text{pH } 3) 226 (1.00) \text{ and } 266 (0.97); (\text{pH } 8 \text{ and } 10) 220 (1.00) \text{ and } 301 (0.53) \]
(Found, after drying at 100° for 6 h: C, 34.4; H, 5.4; N, 28.2; Cl, 25.1. Calc. for \( \text{C}_7\text{H}_11\text{N}_5\text{O}, 1.74 \text{HCl: C, 34.4; H, 5.25; N, 28.6; Cl, 25.2%}. \)

6-Trideuteromethyl-5,6,7,8-tetrahydropterin hydrochloride was prepared by catalytic reduction of 6-trideuteromethylpterin as above. It had m.p. >260° (decomp.)(Found: C, 31.2; H+D, 7.0; Cl, 24.8. \( \text{C}_7\text{H}_8\text{D}_3\text{N}_5\text{O}, 1.9 \text{HCl, H}_2\text{O requires C, 31.0; H+D, 6.65; Cl, 24.7%}. \)
The p.m.r. spectra are in Fig. 2.4.b and 2.5 and in
the Table 2.2. Similarly cis- and trans-7-deutero-6-methyl-5,6,7,8-tetrahydropterin hydrochloride, m.p. >260° (decomp.) (Found: C, 30.5; H+D, 6.1; Cl, 22.9. \( \text{C}_{7}\text{H}_{10}\text{DN}_{5} \cdot 1.8 \text{HCl}, 1.5 \text{H}_{2}\text{O} \) requires C, 30.7; H+D, 6.2; Cl, 23.0%) and cis- and trans-7-deutero-6-trideuteromethyl-5,6,7,8-tetrahydropterin hydrochloride, m.p. >260° (decomp.) (Found: C, 34.1; H+D, 6.6; Cl, 23.6. \( \text{C}_{7}\text{H}_{7}\text{D}_{4}\text{N}_{5} \cdot 1.6 \text{HCl} \) requires C, 34.4; H+D, 6.8; Cl, 23.7%) with p.m.r. spectrum in Fig. 2.4.c and Table 2.2, were prepared by catalytic reduction as the above. The u.v. spectra and t.l.c. behaviour of these salts were identical with those of authentic 6-methyl-5,6,7,8-tetrahydropterin hydrochloride. The HCl and H\(_2\)O contents of the crystals varied with the drying conditions and the nitrogen analyses were all consistently about 1% too low.

6,7-Dimethylpterin. \(^{141}\) - 2,4,5-Triaminopyrimidin-6(1H)-one (10.0 g), dissolved in boiling water (1100 ml) containing sodium pyrosulphate (\( \text{ca.} 1 \text{ g} \)), was treated dropwise, at just below the boiling point, with biacetyl (12 ml; 3.3 mol. equiv.). A yellow solid started to crystallize out almost immediately and the almost boiling solution was allowed to cool to room temperature overnight. The solid was collected by centrifugation, washed with water, then ethanol and dried to give 7.00 g (88%) of pterin. It was converted into the sodium salt (82%) by recrystallization from 2\( \text{N} \)-sodium hydroxide (150 ml). The salt was washed with a little cold water then ethanol and dried at 100°, and had m.p. >360° (decomp.) \( \delta(\text{CF}_3\text{COOH}) 2.85 \) and 2.82 (no separation but in 2\( \text{N} \)-DCl, \( \Delta \delta 3.2 \) Hz); \( \nu_{\text{max.}} 3440, 3100 \) (NH), 1645 (CO), 1585, 1520, 1460 and 1340;

\( \lambda_{\text{max.}}^{\text{pH} 2} 217 \) (1.00), 251 (0.48), 322 (0.50) and 331 (0.47); (pH 7) 226 (1.00), 269 (0.72) and 356 (0.62); (pH 10) 250 (1.00), 271 sh (0.60) and 357 (0.40) (Found, after drying at 150° for 12 h:...
C, 45.3; H, 3.9; N, 32.6; Na, 11.1. Calc. for $\text{C}_8\text{H}_8\text{N}_5\text{ONa}$: C, 45.1; H, 3.8; N, 32.85; Na, 10.8%). The more soluble 2,4,5-triaminopyrimidin-6(1H)-one chloride was sometimes used instead of the sulphate in order to (a) reduce the amount of solvent, and (b) avoid catalyst poisoning in later reduction.

6,7-Bis(trideuteriomethyl)pterin sodium salt was prepared in 70% yield by heating 6,7-dimethylpterin (250 mg) at 100° in 2N-sodium deuteroxide in deuterium oxide (25 ml) for 24 h in a sealed tube, and isolated as above. It had no proton signals in the p.m.r. spectrum in 2N-sodium deuteroxide and 2N-deuterium chloride. The u.v. spectra and t.l.c. properties were identical with those of authentic non-deuterated 6,7-dimethylpterin (Found: Na, 9.69. $\text{C}_8\text{H}_2\text{D}_7\text{N}_5\text{ONa}$, $\text{H}_2\text{O}$ requires Na, 9.70%).

cis-6,7-Dimethyl-5,6,7,8-tetrahydropterin hydrochloride was prepared by catalytic reduction of 6,7-dimethylpterin following the general procedure. The tetrahydropterin had m.p. >300° (decomp.); the p.m.r. spectrum (100 MHz) is in Figure 2.8.a and in Table 2.2, p. 75; $\nu_{\text{max}}$, 3510, 3240 (NH), 2900 (CH str), 1665, 1660, 1610 and 1560; $\lambda_{\text{max}}$, (pH 1) 265; (pH 3) 227 (0.95) and 266 (1.00); (pH 8) 299; (pH 10) 221 (1.00), 250sh (0.53), 284sh (0.58) and 300 (0.61)(Found, after drying at 100° for 4 h: C, 36.8; H, 6.3; N, 25.5; Cl, 20.0. Calc. for $\text{C}_6\text{H}_{13}\text{N}_5\text{O}_1\text{.47 HCl}$, 0.75 $\text{H}_2\text{O}$: C, 36.6; H, 6.1; N, 26.7; Cl, 19.9%).

cis- and trans-6,7-Dimethyl-5,6,7,8-tetrahydropterin hydrochloride.—To the sodium salt of 6,7-dimethylpterin (426 mg), under dry nitrogen, in boiling ethanol was added sodium until the u.v. spectrum of an aliquot at pH 2 indicated that reduction was
complete. A total of 17 g of sodium was added during a reflux period of 48 h. The solution was cooled in an ice bath and acidified with a solution of ethanolic hydrogen chloride (120 ml) under nitrogen. Sodium chloride was removed by repeated concentration and filtration. The 1:1 mixture of cis- and trans-6,7-dimethyltetrahydropterin hydrochloride (282 mg) obtained from the mother liquors was recrystallized from ethanol containing a little ethanolic hydrogen chloride under nitrogen. The u.v. spectra of the mixture, m.p. >250° (decomp.) were identical with those of the cis-isomer and the p.m.r. spectrum is in Fig. 2.8.c and Table 2.2 (Found: C, 36.2; H, 5.8; Cl, 24.8. C₈H₁₃N₅O, 1.85 HCl, 0.1 H₂O requires C, 36.3; H, 5.7; Cl, 24.8%). Chromatographic separation of the mixture has not yet been achieved.

cis-6,7-Bis(trideuteromethyl)-5,6,7,8-tetrahydropterin hydrochloride had m.p. >300° (decomp.) (Found: C, 35.0; H+D, 7.8; N, 25.1; Cl, 24.6. C₈H₇D₆N₅O, 1.9 HCl, 0.25 H₂O requires C, 34.9; H+D, 7.3; N, 25.4; Cl, 24.6%). It was prepared by catalytic reduction of 6,7-bis(trideuteriomethyl)pterin sodium salt as above and had u.v. spectra and t.l.c. properties identical with those of the authentic non-deuterated salt. The p.m.r. spectrum is in Fig. 2.8.b and Table 2.2.
PART 3

OPTICAL RESOLUTION OF 6-METHYL-5,6,7,8-TETRAHYDROPTERIN HYDROCHLORIDE

3.1 Discussion

In all the reported studies of the phenylalanine hydroxylase system involving 6-methyl-5,6,7,8-tetrahydropterin as a non-natural coenzyme (see p. 27), only the racemic mixture has been used. Kaufman's studies of the activity of the L-stereoisomer [in respect to the chiral centre at C(6)] of tetrahydrofolic acid relative to the racemic mixture indicated that there may be some stereospecific interaction between the enzymes and the cofactors. It should be possible to establish the relative efficiency of the enzymes involved, phenylalanine hydroxylase and dihydropteridine reductase, towards the stereoisomers of tetrahydrofolic acid by a study of the stereospecificity of the enzymes, both separately and together in the mono-oxygenase system, towards the chiral enantiomers of 6-methyl-5,6,7,8-tetrahydropterin. With this aim in mind, an optical resolution of racemic 6-methyl-5,6,7,8-tetrahydropterin hydrochloride was undertaken.

The resolution was achieved in the classical way, i.e. by addition of a suitable optically active acid to the tetrahydropterin base with formation of a mixture of two diastereomeric salts [e.g. (+)base(-)acid and (-)base(-)acid] which could be separated because of a sufficiently large difference.
between their solubilities. In the present case of 6-methyl-5,6,7,8-tetrahydropterin, it was rather difficult to find a resolving agent which would form diastereomeric salts with the tetrahydropterin that could be separated by recrystallization from a suitable solvent. In unsuccessful trial resolutions, Dr W. Pendergast used camphor sulphonic acid derivatives and tartaric acid. However, Dr Armarego obtained partially resolved 6-methyl-5,6,7,8-tetrahydropterin hydrochloride by using one molar equivalent of 2S,3S-(−)-O,O′-dibenzoyltartaric acid in absolute ethanol under a blanket of nitrogen (in order to prevent aerial oxidation of the tetrahydropterin). The less soluble (+)-base(−)-acid diastereomer precipitated on cooling. The tetrahydropterin hydrochloride was liberated from the precipitated salt by addition of 2N-hydrochloric acid, and the tartaric acid was removed by thorough extraction with chloroform. The product (17%), which had a specific rotation ([α]_{435}^{20} = +10° (c 5.8 in 2N-HCl) was shown by u.v. and p.m.r. spectroscopy to be 6-methyl-5,6,7,8-tetrahydropterin. Addition of ether to the mother liquor caused more salt to precipitate. The free tetrahydropterin hydrochloride from this fraction (~ 9%) had a small positive specific rotation ([α]_{435}^{20} = 1°). Evaporation of the mother liquor left a residue from which tetrahydropterin with a specific rotation of approximately −6° was obtained.

In the present work, an improvement of the above resolution procedure was achieved (outlined in Scheme 3.1).
Scheme 3.1  
Optical resolution of 6-methyl-5,6,7,8-tetrahydropterin hydrochloride

It was found that, by mixing one half of a molar equivalent of (+)-6-methyl-5,6,7,8-tetrahydropterin hydrochloride (3) with one molar equivalent of (-)-0.01-D-Tartaric acid, a salt was precipitated, which consisted of two diastereomers with a lower yield of (−)-base (21) than (+)-base (20). A result, almost all the (+)-base (20) was precipitated as such as a diastereomeric salt of di-iodoacetic acid. The two fractions had specific optical rotations in 2N-hydrochloric acid.

\[
\text{HN}^{-}\text{CH}_3 \text{PhCOO} \quad \text{HCOOH}
\]

\[
\text{HN}^{-}\text{PhCOO} \quad \text{HCOOH}
\]

* in 2N-hydrochloric acid.
It was found that, by mixing one half of a molar equivalent of $2S,3S-(-)-O,O'$-dibenzoyltartaric acid with one molar equivalent of racemic 6-methyl-5,6,7,8-tetrahydropterin, a salt was formed which consisted of two diastereomers with a larger difference between their solubility products than the corresponding salt (one base to one acid) described above. As a result, almost all the $(+)$base$-(-)$acid came out of solution on cooling, and the $(-)$base$-(-)$acid precipitated as soon as a little ether was added. The tetrahydropterin hydrochloride from the two fractions had specific rotations of $+26^\circ$ (A) and $-23.5^\circ$ (B) respectively in 2N-hydrochloric acid. Following precipitation with more ether another 13 mg of hydrochloride was obtained. This fraction (C) and a fourth fraction (D), obtained from the residue after evaporation of the solvent, had $[\alpha]_{435}^{20} = 25^\circ$ and $-21^\circ$ respectively. The o.r.d. curves of fraction (A), $(+)$-6-methyl-5,6,7,8-tetrahydropterin hydrochloride, and (C), $(-)$-6-methyl-5,6,7,8-tetrahydropterin hydrochloride, in water and 2N-hydrochloric acid are shown in Figure 3.1.
Figure 3.1
O.r.d. of Enantiomers of 6-Methyl-5,6,7,8-tetrahydropterin hydrochloride

A (+)-6-Methyl-5,6,7,8-tetrahydropterin hydrochloride
B (-)-6-Methyl-5,6,7,8-tetrahydropterin hydrochloride

°

-30-
-20-
-10-
0
+10-
+20-
+30-

λ 580 560 540 520 500 480 460 440 420 400 nm

- °

in H₂O
in 2N-HCl
3.2 Experimental

Optical rotations (error ± 0.002° unless otherwise stated) and o.r.d. data were measured on a Perkin-Elmer 241 MC photoelectric polarimeter using a 0.2 dm cell connected to a thermostat bath (temperature 20°C unless otherwise stated). Concentrations (c) are in g per 100 ml. P.m.r. and u.v. spectra were recorded and melting points obtained as described in Part 2, Experimental (Section 2.7, p. 91).

(+) and (-) 6-Methyl-5,6,7,8-tetrahydropterin hydrochloride.

6-Methyl-5,6,7,8-tetrahydropterin, 1.74 HCl (489 mg; 2 mol.equiv.) was partly dissolved in absolute ethanol (100 ml) under a blanket of nitrogen, and potassium hydroxide (195 mg; 4 mol.equiv.) in absolute ethanol (2 ml) was added. This solution was treated with 2S,3S-(-)-O,O'-dibenzoyltartaric acid monohydrate (376 mg; 1 mol.equiv.) dissolved in absolute ethanol (50 ml), and the mixture was boiled and filtered hot under nitrogen. On cooling a gelatinous solid precipitated. The mixture was stored under nitrogen in the dark overnight at -15°C, and the white gelatinous solid (285 mg, fraction a) was collected, washed with a little ethanol and dried. Dry ether (80 ml) was added to the mother liquor which was then kept under N₂ at -15°C overnight. The white gelatinous precipitate (19 mg, fraction b) was collected, washed with a little ethanol and dried. More dry ether (100 ml) was added to the mother liquor. The same procedure as above gave 35 mg of gelatinous solid (fraction c) and evaporation of the solvent left a white yellow residue (300 mg, fraction d) which was washed and dried.
All the salts (a, b, c and d) became gummy when 2N-aqueous hydrochloric acid was added (2 ml to a, b and c, and 10 ml to d respectively), so the mixtures were shaken vigorously with chloroform (a, b, c, 10 ml; d, 25 ml) on a mechanical shaker for 15 min each. The aqueous layers were extracted further with chloroform (3 x 10 ml for a, b, c; 3 x 25 ml for d) in order to remove all the tartaric acid derivative, and filtered through Celite and charcoal. The optical rotations of the four clear solutions of fraction A, B, C and D respectively and other physical data were as follows:

Fraction A in 2N-hydrochloric acid (~ 2 ml) had:

<table>
<thead>
<tr>
<th>λ (nm)</th>
<th>589</th>
<th>578</th>
<th>546</th>
<th>435</th>
</tr>
</thead>
<tbody>
<tr>
<td>α°</td>
<td>.156</td>
<td>.161</td>
<td>.186</td>
<td>.348</td>
</tr>
</tbody>
</table>

Evaporation of the solvent left 134 mg (27%) of white solid which had m.p. > 240° (decomp.) and p.m.r. and u.v. spectra identical with those of authentic 6-methyl-5,6,7,8-tetrahydropterin hydrochloride (Found: C, 33.3; H, 5.4; N, 27.4; Cl, 27.5. C7H11N5O.1.96 HCl requires C, 33.3; H, 5.2; N, 27.7; Cl, 27.5%).

Fraction A (40.71 mg) was dissolved in 2.00N-HCl (2.00 ml; σ = 2), the optical rotations of the solution were measured and the specific rotations calculated.

<table>
<thead>
<tr>
<th>λ (nm)</th>
<th>589</th>
<th>578</th>
<th>546</th>
<th>435</th>
</tr>
</thead>
<tbody>
<tr>
<td>α°</td>
<td>.046</td>
<td>.048</td>
<td>.057</td>
<td>.105</td>
</tr>
<tr>
<td>[α]°</td>
<td>+11</td>
<td>+12</td>
<td>+14</td>
<td>+26</td>
</tr>
</tbody>
</table>

See Figure 3.1 and later for o.r.d. curves.

Fraction B in 2N-hydrochloric acid (~ 2 ml) had:

<table>
<thead>
<tr>
<th>λ (nm)</th>
<th>578</th>
<th>546</th>
<th>435</th>
</tr>
</thead>
<tbody>
<tr>
<td>α°</td>
<td>-.020</td>
<td>-.022</td>
<td>-.041</td>
</tr>
</tbody>
</table>
Fraction C in 2N-hydrochloric acid (~ 2 ml) had:

<table>
<thead>
<tr>
<th>λ (nm)</th>
<th>578</th>
<th>546</th>
<th>435</th>
</tr>
</thead>
<tbody>
<tr>
<td>α o</td>
<td>-0.033</td>
<td>-0.036</td>
<td>-0.067</td>
</tr>
</tbody>
</table>

Evaporation of the solvent left 20 mg (~ 4%) of white solid with p.m.r. and u.v. spectra identical with the above; m.p. >240°C. 19.34 mg of this fraction was dissolved as above and the following optical and specific rotations were obtained:

<table>
<thead>
<tr>
<th>λ (nm)</th>
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<th>546</th>
<th>435</th>
</tr>
</thead>
<tbody>
<tr>
<td>α o</td>
<td>-0.023</td>
<td>-0.025</td>
<td>-0.048</td>
</tr>
<tr>
<td>[α] o</td>
<td>-12</td>
<td>-13</td>
<td>-25</td>
</tr>
</tbody>
</table>

See Figure 3.1 and later for o.r.d. curves.

Fraction D (~ 25 mg) in 2N-hydrochloric acid (~ 2 ml) had:

<table>
<thead>
<tr>
<th>λ (nm)</th>
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<th>546</th>
<th>435</th>
</tr>
</thead>
<tbody>
<tr>
<td>α o</td>
<td>-0.036</td>
<td>-0.041</td>
<td>-0.075</td>
</tr>
</tbody>
</table>

Evaporation of the solvent from all the solution left 140 mg (~ 29%) of white yellow with u.v. spectra as above; m.p. > 240°C. This fraction (32.13 mg) was dissolved as above and the following optical and specific rotations were obtained:

<table>
<thead>
<tr>
<th>λ (nm)</th>
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<th>546</th>
<th>435</th>
</tr>
</thead>
<tbody>
<tr>
<td>α o</td>
<td>-0.032</td>
<td>-0.035</td>
<td>-0.068</td>
</tr>
<tr>
<td>[α] o</td>
<td>-10</td>
<td>-11</td>
<td>-21</td>
</tr>
</tbody>
</table>

Optical rotation of 2S,3S-(−)-O,O'-dibenzoyltartaric acid

2S,3S-(−)-O,O'-dibenzoyltartaric acid (40.64 mg) was dissolved in ethanol (2.00 ml) and the optical rotation at 589 nm was measured: α = -470° ⇒ [α] 20 589° = -116° (lit., 163 -116.0°).
**O.r.d. data**

(+)-6-Methyl-5,6,7,8-tetrahydropterin hydrochloride (fraction A)

<table>
<thead>
<tr>
<th>λ (nm)</th>
<th>α₀</th>
<th>[α]₀</th>
<th>α₀</th>
<th>[α]₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>590</td>
<td>+0.041</td>
<td>+10</td>
<td>+0.049</td>
<td>+12</td>
</tr>
<tr>
<td>578</td>
<td>+0.041</td>
<td>+10</td>
<td>+0.051</td>
<td>+13</td>
</tr>
<tr>
<td>570</td>
<td>+0.044</td>
<td>+10.5</td>
<td>+0.054</td>
<td>+14</td>
</tr>
<tr>
<td>560</td>
<td>+0.045</td>
<td>+11</td>
<td>+0.056</td>
<td>+14</td>
</tr>
<tr>
<td>550</td>
<td>+0.047</td>
<td>+11</td>
<td>+0.058</td>
<td>+15</td>
</tr>
<tr>
<td>546</td>
<td>+0.049</td>
<td>+12</td>
<td>+0.060</td>
<td>+15</td>
</tr>
<tr>
<td>540</td>
<td>+0.049</td>
<td>+12</td>
<td>+0.062</td>
<td>+15.5</td>
</tr>
<tr>
<td>530</td>
<td>+0.052</td>
<td>+12</td>
<td>+0.064</td>
<td>+16</td>
</tr>
<tr>
<td>520</td>
<td>+0.054</td>
<td>+13</td>
<td>+0.068</td>
<td>+17</td>
</tr>
<tr>
<td>510</td>
<td>+0.056</td>
<td>+13.5</td>
<td>+0.070</td>
<td>+18</td>
</tr>
<tr>
<td>500</td>
<td>+0.060</td>
<td>+14</td>
<td>+0.074</td>
<td>+19</td>
</tr>
<tr>
<td>490</td>
<td>+0.063</td>
<td>+15</td>
<td>+0.078</td>
<td>+20</td>
</tr>
<tr>
<td>480</td>
<td>+0.067</td>
<td>+16</td>
<td>+0.084</td>
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</tr>
<tr>
<td>470</td>
<td>+0.071</td>
<td>+17</td>
<td>+0.088</td>
<td>+22</td>
</tr>
<tr>
<td>460</td>
<td>+0.076</td>
<td>+18</td>
<td>+0.093</td>
<td>+23</td>
</tr>
<tr>
<td>450</td>
<td>+0.081</td>
<td>+19</td>
<td>+0.100</td>
<td>+25</td>
</tr>
<tr>
<td>440</td>
<td>+0.086</td>
<td>+20.5</td>
<td>+0.107</td>
<td>+27</td>
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<tr>
<td>435</td>
<td>+0.089</td>
<td>+21</td>
<td>+0.110</td>
<td>+28</td>
</tr>
<tr>
<td>430</td>
<td>+0.093</td>
<td>+22</td>
<td>+0.114</td>
<td>+29</td>
</tr>
<tr>
<td>425</td>
<td>-</td>
<td>-</td>
<td>+0.118</td>
<td>+30</td>
</tr>
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</table>

12.26 mg in 5.00 ml EtOH

<table>
<thead>
<tr>
<th>λ (nm)</th>
<th>α₀</th>
<th>[α]₀</th>
<th>α₀</th>
<th>[α]₀</th>
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<tbody>
<tr>
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<td>+20</td>
<td>390</td>
<td>+0.014</td>
</tr>
<tr>
<td>420</td>
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<td>+26</td>
<td>370</td>
<td>+0.014</td>
</tr>
<tr>
<td>410</td>
<td>+0.013</td>
<td>+26</td>
<td>365</td>
<td>+0.018</td>
</tr>
<tr>
<td>400</td>
<td>+0.014</td>
<td>+28</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
(-)-6-Methyl-5,6,7,8-tetrahydropterin hydrochloride (fraction C)

17.62 mg in 2.00 ml H₂O

19.34 mg in 2.00 ml 2.00N-HCl

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4.1 Introduction

A knowledge of the absolute configurations of the enantiomeric 6-methyl-5,6,7,8-tetrahydropterins is required for a complete understanding of the stereospecificity of phenylalanine hydroxylase and dihydropteridine reductase in the mono-oxygenase enzyme system. In order to obtain this knowledge it would be necessary to degrade the pyrimidine ring of at least one of the previously resolved stereoisomers of 6-methyl-5,6,7,8-tetrahydropterin without affecting the asymmetric centre at C(6). It should then be possible to establish the configuration at C(6) [C(2) in the resulting piperazine ring] by comparing its chiroptical properties with those of a similar piperazine of known absolute configuration at C(2) [see Part 5]. However, it is important at first to find means of stabilizing 6-methyl-5,6,7,8-tetrahydropterin towards oxidation because, like most tetrahydropterins, it is oxidized readily by air to 6-methyl-7,8-dihydropterin in which the chiral centre at C(6) is lost.

The present Part includes a very brief discussion of the autoxidation of tetrahydropterins, studies on the methylation of 6-methyl-5,6,7,8-tetrahydropterin, and the stability of the methylated derivatives towards aerial oxidation.
4.2 Autoxidation of 5,6,7,8-tetrahydropterins

Kaufman and collaborators\textsuperscript{164} showed that molecular oxygen is incorporated as the hydroxy group in tyrosine when formed from phenylalanine in the mono-oxygenase system (see Scheme 1.4, p.32). The complete mechanism for this incorporation, which has been the subject of much discussion, has not yet been completely established.

It has been suggested\textsuperscript{165-169} that transient hydroperoxide intermediates formed by autoxidation of the tetrahydropterin may be the source of the hydroxy group. It has also been suggested that autoxidation occurs by a chain reaction involving hydroperoxide formation at C(6) and C(7),\textsuperscript{170} or by a free radical chain reaction in which the chain carrier is the hydroperoxyl radical (HOO·).\textsuperscript{171} The different theories are described briefly in the following. A review on this subject has been published by Kaufman and Fischer.\textsuperscript{172}

Following studies involving tritium-labelling, Kaufman\textsuperscript{111} proposed ortho- (4.1) and/or para- (4.2) quinonoid structures for dihydropteridine as the primary oxidation products formed from tetrahydropteridines during both the non-enzymatic and the phenylalanine hydroxylase-catalyzed oxidation. Beforehand, Kaufman had ruled out the possibility of 5,6-, 7,8- and 5,8-dihydro structures. Spectral studies by Hemmerich\textsuperscript{173} supported Kaufman's proposition. Mager and Berends,\textsuperscript{165} however, did not accept the quinonoid structures as the primary products.
They argued that the fact that one atom of the oxygen molecule is incorporated into the substrate and the other reduced to water, indices that the original oxygen molecule is converted, formally by the uptake of one or two or three electrons.

Haynes and collaborators \(^{1/4}\) were of the opinion that the ultimate cleavage of the oxygen-oxygen bond suggests the intermediacy of the \(\text{H}_{\text{2}}\text{O}_{2}\) and postulated that the tetrahydropteridine anion reacts with the \(\text{HO}^{+}\) ion by covalent attachment of the latter to the tetrahydropteridine ring in such a way as to give rise to a hydroxytetrahydropteridine \((4.4)\) carrying the hydroxyl group at the same position. Dehydration would then lead to a quinonoid dihydropteridine \((4.3)\) in Scheme 1.4. The proposal was based on the following considerations: (a) autoxidation of the reduced ring-system of tetrahydropteridines (tetrahydroalloxazines) gives \(\text{HO}^{+}\) ions as an intermediate, (b) autoxidative rearrangements of \(\text{N}\)-substituted dihydropteridines (tetrahydroalloxazines) which rearranged when oxidized. At least two reactions caused a conversion of the peroxide \((4.3)\) into the quinonoid \((4.4)\): (a) simultaneous oxidation of a separate pteridine hydroperoxide or hydrogen peroxide and (b) hydrolytic formation of hydrogen peroxide and subsequent reaction of the cationic species \((4.5)\) with the reactions (a) and (b) could take place simultaneously. However,
They argued that the fact that one atom of the oxygen molecule is incorporated into a substrate and the other reduced to water, indicates that the original oxygen molecule is converted into a more reactive species, formally by the uptake of one, two or three electrons. Mager and collaborators\textsuperscript{174} were of the opinion that the ultimate cleavage of the oxygen-oxygen bond suggests the intermediacy of peroxides, and postulated that the tetrahydropteridine causes an 'activation' of the oxygen by covalent linking of the latter to a bridgehead carbon. Both C(4a) and C(8a) may be involved in the reaction. Preference was given to the 8a-position, expecting that such a tetrahydropteridine hydroperoxide (4.3) could give rise to a hydroxytetrahydropteridine (4.4) carrying the hydroxyl group at the same position. Dehydration would then lead to a quinonoid dihydropteridine [\textit{cf.} (1.44) in Scheme 1.4, p.32]. This proposal was based upon studies on autoxidation of some related ring-systems (e.g. tetrahydropyrazines and dihydroalloxazines) and on autoxidative rearrangements of N-substituted (blocked) tetrahydropteridines (lumazines) which rearranged when oxidized. At least two reactions caused a conversion of the peroxide (4.3) into the hydroxy derivative (4.4; \textit{cf.} Scheme 1.4): (a) simultaneous oxidation of a separate tetrahydropterin molecule by the tetrahydropterin hydroperoxide or hydrogen peroxide, and (b) hydrolytic formation of hydrogen peroxide and subsequent reaction of the cationic species (4.5) with water. The reactions (a) and (b) could take place simultaneously. However,
suitable conditions could be found to favour each of the reactions, resulting in varying oxygen-uptake, net hydrogen peroxide production and oxygen-transfer. In particular, an attempt was made to promote competitive oxidation as chemical imitations of enzymatic mono-oxygenations. It was thus proved that the non-enzymatic hydroxylation of phenylalanine is accomplished by radicals (e.g. HO·) arising from an organic peroxo transient. In support of the existence of a peroxo transient, Mager and Berends reported the isolation of an explosive benzoyl derivative of tetrahydropterin hydroperoxide. This had been obtained by careful benzoylation of the product from the rapid autoxidation of 6,7-dimethyl-5,6,7,8-tetrahydropterin.

Mager and collaborators have contributed greatly to the discussion of whether the 4a- or the 8a-bridgehead carbon is involved in the autoxidation of tetrahydropteridines. They found, for example, that 4a-hydroxytetrahydrodrolumazines are not formed as final products in the autoxidation of 1,3,5-trimethyl- and 1,3,6,7,8-pentamethyl-tetrahydrodrolumazine but that unexpected ring-contractions occur in which either the original C(4a)- or C(8a)-atom becomes the spiro centre (see Scheme 4.1; for further reading see reference).

Another school led by Blair does not assume the intermediacy of a tetrahydropteridine hydroperoxide in the formation of the quinonoid dihydropteridines and the hydroperoxy radical. Based on product analysis and a kinetic study of the autoxidation of tetrahydrobiopterin and tetrahydrofolic acid, Blair and Pearson proposed
Autoxidation of 1,3,6,7,8-pentamethyl-5,6,7,8-tetrahydrolumazine
a mechanism involving oxygen attack at C(4a), and implicated the hydroperoxy radical as chain carrier (see Scheme 4.2). Blair, Pearson and Robb\textsuperscript{178} reported a similar study of the related compound 5-methyl-5,6,7,8-tetrahydrofolic acid (the major mammalian folate monoglutamate), which Gapski, Whiteley and Huennekens\textsuperscript{179} had shown may be oxidized by hydrogen peroxide to 4a-hydroxy-5-methyl-4a,5,6,7-tetrahydrofolic acid and 5-methyl-5,6-dihydrofolic acid. For this conversion Blair \textit{et al.}\textsuperscript{178} proposed the mechanism of autoxidation shown in Scheme 4.3. It was found that copper(II) ions at low concentrations accelerated the abovementioned reaction. The apparent specificity for copper catalysis indicated that this was not due to chain initiation but rather a reduction of copper(II) to copper(I) by the tetrahydropterins and by the free radicals (e.g. 4.6) followed by a rapid reoxidation to copper(II). The overall reactions were found to be first order in oxygen with a linear dependence of rate on the percentage ionization of the 3,4-amide group.\textsuperscript{171} The 5-methyl derivative was autoxidized approximately ten times more slowly than was tetrahydrofolic acid itself. This was consistent with the proposal\textsuperscript{178} that oxygen attack occurred at C(4a) where the presence of an N(5)-substituent would lead to either steric hindrance to oxygen approach or steric compression in any transition state for electron transfer from the reduced compound or radical (4.6) to oxygen or to a chain carrier. An increase in pH was shown to decrease the rate of autoxidation of the above
INITIATION

(a) electron withdrawal
(b) proton loss

PROPAGATION

(II) + O₂  \rightarrow \begin{array}{c}
\text{(a) electron transfer} \\
\text{(b) rapid proton transfer}
\end{array}

(I) + HO₂⁻  \rightarrow \begin{array}{c}
\text{(II) + H₂O₂}
\end{array}

Scheme 4.2 Mechanism of autoxidation of tetrahydropterins as proposed by Blair and Pearson.
Mechanism of autoxidation of 5-methyl-5,6,7,8-tetrahydrofolic acid as proposed by Blair, Pearson and Robb (ref.178).
tetrahydro compounds considerably; e.g. no reaction could be measured at pH values below 4.

Blair and Pearson\textsuperscript{171} also proposed a mechanism for the autooxidative ring-contraction of 1,3,6,7,8-pentamethyl-5,6,7,8-tetrahydrolumazine. The suggested reaction did not involve the intermediacy of an organic hydroperoxide. The radical cation was expected to react with water, instead of with the pterinium cation, followed by a second one-electron reduction of the hydroperoxy radical to hydrogen peroxide. Mager \textit{et al}.\textsuperscript{174} argued against the theory; e.g. because the mechanism did not explain the formation of spirohydantoins in non-protic solvents (e.g. acetonitrile; see Scheme 4.1).

Jaenicke and Wahlefeld\textsuperscript{180,181} investigated the oxidation of 6,8-dimethyl-7,8-dihydropterin and reported the reactions which are schematically presented in Scheme 4.4.a. They postulated the involvement of a dihydropterin radical (4.7) as shown in Scheme 4.4.b.

Viscontini and his collaborators have studied the aerial oxidation of tetrahydropteridines over several years. Viscontini and Okada\textsuperscript{182} proposed a 4a-hydroxytetrahydropterin structure for the product from the autoxidation of 5-methyl-6,7-diphenyl-5,6,7,8-tetrahydropterin at pH $>$ 7, and discussed the involvement of iron(II)/iron(III) complexes in the conversion.\textsuperscript{183} Several authors\textsuperscript{184} have since assumed that the $\text{C}(4a)$-$\text{C}(5)$-bond in 6,7-dihydropteridine species is hydrated to give 4a-hydroxytetrahydropteridines analogous to the stable
Scheme 4.4.a Oxidation of 6,8-dimethyl-7,8-dihydropterin (from ref.180)

- Pt/H₂, 25°, acetic acid
- O₂ or K₃[Fe(CN)₆]
- pH 7.0, 20 min

- KMnO₄/NaOH, 25°, 3 days
- O₂, pH 7.0
- pH 7.0, 20 min

- Scheme 4.4.b
Scheme 4.4.b
derivative isolated by Viscontini and Okada. Later, however, Viscontini and Argentini\textsuperscript{185} demonstrated that the pyrazine ring of tetrahydropterins could be opened during aerial oxidation, to yield 2-amino-alloxan\textsuperscript{186} which proved that the proposed 4a-hydroxy structure was wrong.

Hamilton\textsuperscript{187} postulated the involvement of the 4a-bridgehead-carbon in the ring opening of 4a-hydroperoxy-4a,5,6,7-tetrahydropterin which after loss of an oxygen atom rapidly reclosed to give 4a-hydroxy-4a,5,6,7-tetrahydropterin. It was meant that this reaction sequence proceeded within an iron complex at the active site. It was, however, also thought that the metal ion (in enzymatic reactions) is not involved in the usual catalytic reaction but only in the protection of the enzyme from inactivation. However, the role of iron is not yet clear.\textsuperscript{172}

The question of whether the 4a- or the 8a-bridgehead carbon is involved in the oxidation of tetrahydropterin is still a subject for verification, although it appears to be generally accepted that the 4a-position is involved in the case of 5,6,7,8-tetrahydropterins. In non-enzymatic reactions, Mager's study of irreversible reactions with lumazines\textsuperscript{174} showed that oxygen can be covalently linked to either C(4a) or C(8a) or perhaps to both. For tetrahydropterin-dependent enzymatic oxygenations, Hemmerich\textsuperscript{174,188} postulated that position 4a (and not 8a) was uniquely involved. Kaufman,\textsuperscript{189} who has been very active in the biological aspects of this field, has
suggested from u.v. spectral evidence that 4a-hydroxy species are formed in biologically relevant oxygenations (involving biopterin). However, until further evidence is presented, it still remains an assumption that 4a-hydroperoxy- and 4a-hydroxy-tetrahydropterins are indeed intermediates.

With the present project in mind, it is of special interest to note that the rates by which 5,6,7,8-tetrahydropterins undergo aerial oxidation are dependent on the pH of the solution, and on the extent of substitution. Matsuura and Sugimoto found that the 5,6,7,8-tetrahydropterin anion decomposed within 15 minutes in solution at pH 14 and 25°, whereas under the same conditions 5-methyl-5,6,7,8-tetrahydropterin decomposed slowly during 30 hours. The same stabilizing effect was observed for the neutral molecules. Non-enzymatic oxidation of methyl derivatives of 5,6,7,8-tetrahydropterin generally yielded the 7,8-dihydro derivatives.

In order to stabilize 6-methyl-5,6,7,8-tetrahydropterin, a study of the methylation of this compound was undertaken.

4.3 Methylation of 5,6,7,8-tetrahydropterins

An attempt to methylate a non-reduced pterin, 6,7-dimethylpterin, by treatment with methyl iodide
in N,N-dimethylformamide in a sealed tube on a steam bath for 12 hours was unsuccessful. Likewise unsuccessful was treatment of the pterin with dimethyl sulphate in basic solution, and treatment with methyl iodide in liquid ammonia. The starting material was recovered after each attempted methylation. 5,6,7,8-Tetrahydopterins, on the other hand, were found to be methylated quite readily by methyl iodide in basic solution as described in the following section.

4.3.1 Methylation of 6-methyl-5,6,7,8-tetrahydopterin

Catalytic reduction of 6-methylpterin in an acidic medium gave 6-methyl-5,6,7,8-tetrahydopterin which was isolated as a hydrochloride (see page 121). The molar ratio of hydrogen chloride to tetrahydopterin in the product was always greater than one and nearer to two. The ratio depended on the extent to which the salt was heated in a vacuum, but it did not alter when the salt was stored in the dark. Most of the present work was performed on the hydrochlorides of 6-methyl-5,6,7,8-tetrahydopterin which contained between one and two molar proportions of hydrogen chloride.

Armarego\textsuperscript{194} found that a trimethyl-5,6,7,8-tetrahydopterin was formed when a methanolic solution of 6-methyl-5,6,7,8-tetrahydopterin hydrochloride containing three molar equivalents of sodium hydroxide and an excess of methyl iodide (10 mol. equiv.) was boiled for three hours in a nitrogen atmosphere. The product was isolated as the hydrochloride after
purification through a Dowex 50W column by elution with 3N-hydrochloric acid. If, instead of isolating the product, the solution (which had become neutral after boiling because of hydrolysis of the methyl iodide) was treated further with sodium hydroxide (3 mol. equiv.) and an excess of methyl iodide (10 mol. equiv.), and then boiled for another three hours, a tetramethyl-5,6,7,8-tetrahydropterin was formed. The product was isolated as the hydrochloride by ion exchange chromatography as the previous trimethyl derivative. A third treatment with methyl iodide and sodium hydroxide did not cause any additional methylation. On the other hand, one methyl group was lost when the tetramethyl-5,6,7,8-tetrahydropterin was eluted with 3N-aqueous ammonia from a Dowex 50W column.

Armarego reasoned at first that, in the presence of at least one molar excess of alkali, the anion (4.8) of the 6-methyl-5,6,7,8-tetrahydropterin was formed, and on methylation yielded 3,6-dimethyl-5,6,7,8-tetrahydropterin (4.9). Because the most basic centre in 5,6,7,8-tetrahydropterins is N(5), subsequent methylation would proceed to give 3,5,6-trimethyl-5,6,7,8-tetrahydropterin (4.10). Further treatment with methyl iodide and sodium hydroxide would probably cause the trimethyl derivative (4.10) to undergo a Dimroth rearrangement to 5,6-dimethyl-2-methylamino-5,6,7,8-tetrahydropteridin-4(3H)-one (4.11) which would form an anion and would be methylated further to 3,5,6-trimethyl-2-methylamino-5,6,7,8-tetrahydropteridin-4(3H)-one (4.12).
Purification of the tetramethyl derivative (4.8) through a Dowex 50w column by elution with 3M aqueous hydrochloric acid would cause displacement of the 2-methylamino group by ammonia to give the tetramethyl derivative (4.9). Treatment of 4.8 with sodium ethoxide followed by reaction with methyl bromide and sodium hydroxide. This gave a 1:1 mixture of a tetramethyl derivative identical with the above, together with a dimethyltetrahydropterin which was identified as 6-methyl-2-methylamino-5,6,7,8-tetrahydropteridin-4(1H)-one (4.10). An authentic sample of the hydrochloride was prepared by catalytic reduction of 3,6-dimethylpterin to 3,6-dimethyl-5,6,7,8-tetrahydropterin and conversion into 5-formyl-3,6-dimethyl-5,6,7,8-tetrahydropterin (4.14) with a mixture of ethyl acetate and acetic anhydride. It is known that formylation of the 5,6,7,8-tetrahydropterins by this method takes place on N(5), and that the formyl derivative is protonated on the formyl oxygen atom to give a cation similar to 4.12. (See Experimental, p. 175 for further details). The p.m.r. spectrum of the formyl derivative (4.14) in trifluoroacetic acid...
Purification of the tetramethyl derivative (4.12) through a Dowex 50W column by elution with 3N-aqueous ammonia would cause displacement of the 2-methylamino group by ammonia to give the trimethyl derivative (4.10).

To test the above reasoning, Armarego reduced authentic 3,6-dimethylpterin to 3,6-dimethyl-5,6,7,8-tetrahydropterin and methylated it with methyl iodide and sodium hydroxide. This gave a 1:1 mixture of a tetramethyl derivative identical with the above, together with a dimethyltetrahydropterin which was identified as 6-methyl-2-methylamino-5,6,7,8-tetrahydropteridin-4(3H)-one (4.13). An authentic sample of the hydrochloride of the latter was prepared by reducing 6-methyl-2-methylaminopteridin-4(3H)-one which was previously prepared by a Dimroth rearrangement of 3,6-dimethylpterin. This experiment established that at least one methyl group in the above tetramethyl derivative was on N(3). Authentic 3,5,6-trimethyl-5,6,7,8-tetrahydropterin was prepared as the hydrochloride by catalytic reduction of 3,6-dimethylpterin to 3,6-dimethyl-5,6,7,8-tetrahydropterin, and conversion into 5-formyl-3,6-dimethyl-5,6,7,8-tetrahydropterin (4.14) with a mixture of formic acid and acetic anhydride. It is known that formylation of 5,6,7,8-tetrahydropterins by this method takes place on N(5), and that the formyl derivative is protonated on the formyl oxygen atom to give a cation similar to 4.15. (See Experimental, p. 178 for further details). The p.m.r. spectrum of the formyl derivative (4.14) in trifluoroacetic acid
was consistent either with a mixture of two isomers (syn and anti) of the cation (4.15), or a mixture of the neutral species (4.14) and the cation (4.18). Catalytic reduction of tetrahydrofolic acid to the 5,6,7,8-tetrahydro-5,6,7,8-tetramethylpterin has been reported. The authentic derivative (4.10), however, was not identical with the trimethyl-5,6,7,8-tetrahydropterin isolated following direct methylation of 5-formyl-6,7-dimethyl-5,6,7,8-tetrahydropterin, or from trimethyl-6-methyl-5,6,7,8-tetrahydropterin, or from trimethyl-5,6,7,8-tetrahydropterin hydrochloride (4.16). Methylation of this salt in the presence of alkali gave a trimethyl derivative identical with the trimethyl derivative obtained by direct methylation of 6-methyl-5,6,7,8-tetrahydropterin, proving that the trimethyl derivative was 1,6-dimethyl-5,6,7,8-tetrahydropterin chloride (4.16). It is noteworthy that the trimethyl derivative obtained from using the tetramethyl derivative with Dowex 50W-X8-aqueous ammonia was a lunazine, formed from loss of a 2-methylamino group from 3,5,6-trimethyl-2-methylamino-5,6,7,8-tetrahydropteridin-4(3H)-one, was excluded by the preparation of authentic
was consistent either with a mixture of two isomers (syn and anti) of the cation (4.15), or a mixture of the neutral species (4.14) and the cation (4.15). Catalytic reduction of the formyl derivative (4.14) in trifluoroacetic acid gave the authentic 3,5,6-trimethyl-5,6,7,8-tetrahydropterin (4.10). A similar reduction of 5-formyl-6,7-dimethyl-5,6,7,8-tetrahydropterin to 5,6,7-trimethyl-5,6,7,8-tetrahydropterin has been reported.\(^{153}\) The authentic derivative (4.10), however, was not identical with the trimethyl-5,6,7,8-tetrahydropterin isolated following direct methylation of 6-methyl-5,6,7,8-tetrahydropterin, or from treatment of the tetramethyl-5,6,7,8-tetrahydropterin with Dowex 50W-3N-aqueous ammonia.

Armarego and Milloy\(^{196}\) reported that catalytic reduction of 1,6-dimethylpterin gave 1,6-dimethyl-5,6,7,8-tetrahydropterin hydrochloride. Methylation of this salt in the presence of alkali gave a trimethyl derivative identical with the trimethyl derivative obtained by direct methylation of 6-methyl-5,6,7,8-tetrahydropterin, and established that the trimethyl derivative was 1,3,6-trimethyl-5,6,7,8-tetrahydropterinium chloride (4.16).\(^{194}\) The possibility that the trimethyl derivative obtained from treating the tetramethyl derivative with Dowex 50W-3N-aqueous ammonia was a lumazine, formed from loss of a 2-methylamino group from 3,5,6-trimethyl-2-methylamino-5,6,7,8-tetrahydropteridin-4(3\(H\))-one, was excluded by the preparation of authentic
by catalytic reduction of 1,3,6-trimethyl-5,6,7,8-tetrahydropteridine (4.18). No other methods, namely, the hydrogenation method, were found to be useful for the preparation of 1,3,6-trimethyl-5,6,7,8-tetrahydropteridine or any of its methylated derivatives. The following scheme and the procedures of Hirs and his co-workers [35] were used for the preparation of the above tetrahydropteridine derivative which was obtained from any of the 5,6,7,8-tetrahydropteridine hydrochlorides previously. It was found to be 1,3,6-trimethyl-2-methylamino-4(3H)-oxo-5,6,7,8-tetrahydropteridinium chloride (4.19) hydrochloride by microanalysis and u.v. and p.m.r. spectroscopy. It was observed that a tetramethyl derivative from methylation of 1,3,6-trimethyl-5,6,7,8-tetrahydropteridine did not possess a methyl group on the imino nitrogen atom and the fourth methyl group, therefore, was shown to be on N(5) or N(10). The presence of one methyl group on H(18) in the tetramethyl derivative from methylation of 1,3,6-trimethyl-5,6,7,8-tetrahydropteridine was excluded because similar methylation of 1,3,6-trimethyl-5,6,7,8-tetrahydropteridine hydrochloride and 5,6,7,8-tetrahydropteridinium chloride (4.17) hydrochloride.

This is confirmed in the present work by obtaining a tetramethyl derivative identical with the above tetrahydropteridinium chloride (4.17) hydrochloride through methylation of the hydrochloride of 1,3,6-trimethyl-5,6,7,8-tetrahydropteridine. The latter was prepared in
1,3,6-trimethyl-5,6,7,8-tetrahydrolumazine (4.18), by catalytic reduction of 1,3,6-trimethylllumazine,\textsuperscript{196} and by the different C:N ratio of the microanalytical results. When Armarego\textsuperscript{194} methylated the hydrochloride of 6-methyl-2-methylamino-4(3\textalpha{H})-oxo-5,6,7,8-tetrahydropteridine with methyl iodide and sodium hydroxide, he obtained a tetramethyl derivative which was different from any of the tetramethyltetrahydropterins prepared previously. It was found to be 1,3,6-trimethyl-2-methylamino-4(3\textalpha{H})-oxo-5,6,7,8-tetrahydropteridinium chloride (4.19) hydrochloride by microanalysis and u.v. and p.m.r. spectroscopy. It was deduced that, the tetramethyl derivative from methylation of 6-methyl-5,6,7,8-tetrahydropterin did not possess a methyl group on the exocyclic nitrogen atom, and the fourth methyl group must therefore have been on N(5) or N(8). The presence of a methyl group on N(8) in the tetramethyl derivative from methylation of 6-methyl-5,6,7,8-tetrahydropterin was excluded because similar methylation of 6,8-dimethyl-5,6,7,8-tetrahydropterin hydrochloride gave a pentamethyltetrahydropterin. The data at that stage indicated that the tetramethyl derivative must have been 1,3,5,6-tetramethyl-5,6,7,8-tetrahydropteridinium chloride (4.17) hydrochloride. This is confirmed in the present work by obtaining a tetramethyl derivative identical with the above tetrahydropteridinium chloride (4.17) hydrochloride through methylation of the hydrochloride of 5,6-dimethyl-5,6,7,8-tetrahydropterin. The latter was prepared in
this work by treatment of 6-methyl-5,6,7,8-tetrahydropterin with formaldehyde followed by reduction of the resulting intermediate with potassium borohydride. The p.m.r. and u.v. spectra of the tetramethyl derivative from 6-methyl-5,6,7,8-tetrahydropterin and from 5,6-dimethyl-5,6,7,8-tetrahydropterin are identical (see Figure 4.3.b, p.165), but the i.r. spectra are not completely superimposable even after extensive drying. The identity of the compounds was confirmed beyond doubt by the proton-decoupled $^{13}$C n.m.r. spectra (Figure 4.1.a) in which all ten carbon signals are superimposable. The carbon signal from $\text{CH}_3(6)$ is broad and of low intensity, probably due to ineffective proton-decoupling. In the proton-coupled $^{13}$C n.m.r. spectrum (Figure 4.1.b), however, it appears as a sharp quartet. The assignments of all the signals in the proton-decoupled spectrum are deduced by inspection, by the multiplicity of the signals in the proton-coupled spectrum and by comparison with the signals previously assigned for 5-formyl-6,7-dimethyl- and 5,6,7-trimethyl-5,6,7,8-tetrahydropterin (see Table 4.0). The data confirm that the derivatives 1,3,6-trimethyl- and 1,3,5,6-tetramethyl-5,6,7,8-tetrahydropterinium chloride are pterins and that no skeletal rearrangement had occurred during methylation.

The methylation of 5,6-dimethyl-5,6,7,8-tetrahydropterin turned out to be more complicated than appeared at first. However, before presenting the results of the methylation experiments and the suggested reaction mechanism, the ultraviolet and p.m.r. properties of the
Figure 4.1 67.89 MHz $^{13}$C n.m.r. spectrum of 1,3,5,6-tetramethyl-5,6,7,8-tetrahydropterinium chloride hydrochloride: (a) proton-decoupled; (b) proton-coupled.
Table 4.0

$^{13}$C n.m.r. of 1,3,5,6-tetramethyl-5,6,7,8-tetrahydropterinium chloride (I), 5-formyl-6,7-dimethyl- (II) and 5,6,7-trimethyl-5,6,7,8-tetrahydropterin (III) hydrochlorides

<table>
<thead>
<tr>
<th></th>
<th>I**</th>
<th>II**</th>
<th>III**</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH$_0$(5)</td>
<td>-</td>
<td>161.0</td>
<td>-</td>
</tr>
<tr>
<td>C(4)</td>
<td>154.9</td>
<td>158.0</td>
<td>157.0</td>
</tr>
<tr>
<td>C(2)</td>
<td>150.8</td>
<td>153.1</td>
<td>153.8</td>
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<tr>
<td>C(8a)</td>
<td>145.8</td>
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<tr>
<td>C(4a)</td>
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<td>89.2</td>
<td>89.8</td>
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<td>C(6)</td>
<td>68.6</td>
<td>42.3</td>
<td>58.2</td>
</tr>
<tr>
<td>CH$_3$(3)</td>
<td>54.9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CH$_3$(1)</td>
<td>50.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C(7)</td>
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<td>49.3</td>
<td>42.9</td>
</tr>
<tr>
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<td>-</td>
<td>45.2</td>
</tr>
<tr>
<td>CH$_3$(7)</td>
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<td>15.5</td>
</tr>
<tr>
<td>CH$_3$(6)</td>
<td>13.4</td>
<td>10.8</td>
<td>10.0</td>
</tr>
</tbody>
</table>

* Values recorded in 0.5N-DCI/D$_2$O; see p.181

** Values recorded in DMSO by Bieri and Viscontini. 153
abovementioned methyl derivatives will be described in order to give background data on which the proposed mechanism is based.

4.3.2 Ultraviolet and p.m.r. spectra of 5,6,7,8-tetrahydropterins

The 5,6,7,8-tetrahydropterins have three pKₐ values, one acidic and two basic.¹⁷⁶,¹⁷⁷,¹⁹⁷ The lowest pKₐ of each tetrahydropterin was in the region of -0.5 to 3.0, so the compounds were obtained as hydrochlorides when crystallized from 4N-hydrochloric acid. Because the anion of tetrahydropterins lacking a substituent on N(5) was unstable, no accurate acidic pKₐ was obtained.¹⁹⁷ The two basic pKₐ values were in the region of 4.5 to 6.0 and 10.0 to 11.5.

Four distinct ultraviolet spectra were obtained for each 5,6,7,8-tetrahydropterin depending on the pH of the solution (see Figure 4.2). The spectrum in strong acid is due to the diprotonated species (4.20). The spectrum of the mono-cation (4.21) and of the di-cation are very similar. They both consist of one or two bands below 270 nm (usually at ca. 217 and 265 nm). The neutral species (4.22) also have one or two bands, one of which is above 280 nm. At pH values above 13, the spectrum is due to the mesomeric anion (4.23). The anions have bands at longer wavelengths than 270 nm as in the neutral species.¹⁷⁶,¹⁷⁷

The ionization constants of the 5,6,7,8-tetrahydropterins involved in the present work were not measured because enough data are available to make reasonable predictions of the pKₐ values.
Figure 4.2

Ultraviolet spectra of 5,6,7,8-tetrahydropterins at various pH
It was found that all tetrahydropterines unsubstituted in the pyridoindene ring show distinctive ultraviolet spectra for the different cations. Anaya et al. reported that the 1.3-dimethyl-6,7,8-tetrahydropterin, 1,3,6-trimethyl-6,7,8-tetrahydropterin, and 2-methylamino-4(3H)-oxo-6,7,8-tetrahydropterin chloride, all have ultraviolet maxima in the pH region 2 to 13 which consist of one band at 340 (4.6 mm) for the mono-cations, the neutral species up to above 13 in order to obtain the neutral species (e.g., 4.24). The spectrum of 3,6-dimethyl-6,7,8-tetrahydropterin could not be measured accurately at pH values above 4 because of rapid spectral changes to the Dimroth rearrangement. In contrast, the uv spectra of 2,3,6-trimethyl-6,7,8-tetrahydropterin and 11 (4.23) were the same, and the spectrum at the latter pH value was unchanged after 2 hours (i.e., no Dimroth rearrangement).

The p.m.r. spectra at 60 MHz are all measured under the same conditions in deuterium chloride in deuterium oxide (see Experimental) and the species in solution are probably all protonated H(5), and also to a large extent in the case of 4.24. Spectra in 2H-deuterium chloride and their assignments did not differ significantly from those in neutral acid. Analyses of the ABC (AA'BB') type coupling pattern of signals for 9(6) and the two protons on C(7) (for Figure 4.3b) were not attempted because these are too complex.
It was found that all tetrahydropterins unsubstituted in the pyrimidine ring show four distinctive ultraviolet spectra for the different species. Armarego reported that the 1,3-dimethyl-5,6,7,8-tetrahydropterinium salts, \textit{viz.} 1,3,6-trimethyl- and 1,3,5,6-tetramethyl-5,6,7,8-tetrahydropterinium chloride, and 1,3,6-trimethyl-2-methylamino-4(3H)-oxo-5,6,7,8-tetrahydropteridinium chloride, all have ultraviolet spectra in the pH region 2 to 13 which consist of one band (at \textit{ca.} 266 nm) for the mono-cation; the pH had to be increased to above 13 in order to obtain the neutral species (e.g. 4.24). The spectrum of 3,6-dimethyl-5,6,7,8-tetrahydropterin could not be measured accurately at pH values above 4 because of rapid spectral changes caused by the Dimroth rearrangement. In contrast, the u.v. spectra of 3,5,6-trimethyl-5,6,7,8-tetrahydropterin at pH 8 and pH 13 were the same, and the spectrum at the latter pH value was unchanged after 2 hours (i.e. no Dimroth rearrangement).

The p.m.r. spectra at 60 MHz are all measured under the same conditions in 0.5\textsubscript{N}-deuterium chloride in deuterium oxide (see Experimental) and the species in solution are probably all protonated at N(5), and also to a large extent in the pyrimidine ring. Spectra in 2\textsubscript{N}-deuterium chloride in deuterium oxide did not differ significantly from those in more dilute acid. Analyses of the ABC (AA'\textit{B}) type coupling pattern of signals for H(6) and the two protons on C(7) (\textit{cf.} Figure 4.3.b) were not attempted because these are too complex
(see the computer-simulated spectrum for 6-methyl-5,6,7,8-tetrahydropterin on p.71). All the following comparisons are made from the assignments of the methyl signals. The methyl group at C(6) always appears as a doublet at high field (ca. 2.1 p.p.m.) and is a useful probe in all the compounds. Methyl groups on the nitrogen atoms are sharp signals. Methyl groups on N(1) and N(3) in the pterinium salts are at low field (δ~ 4.0 p.p.m.) and the lowest-field N-methyl signal was found in 1,3,5,6-tetramethyl-5,6,7,8-tetrahydropterinium chloride. (See Section 4.3.3 for assignments of the three N-methyl signals of the last-mentioned salt.)

The N-methyl signal at lowest field in 1,3,6-trimethyl-5,6,7,8-tetrahydropterinium chloride was assigned to N(1) because the signals at lowest field in 3,6-dimethyl- and 3,5,6-trimethyl-5,6,7,8-tetrahydropterin are at higher field than those in 1,6-dimethyl- and 1,5,6-trimethyl-5,6,7,8-tetrahydropterin respectively. The N(5)-methyl signals in 5,6-dimethyl-, 1,5,6-trimethyl- and 3,5,6-trimethyl-5,6,7,8-tetrahydropterin, and the exocyclic methyl group on NH₂(2) are at δ~ 3.6 p.p.m.

4.3.3. Methylation of 5,6-dimethyl-5,6,7,8-tetrahydropterin

Methylation of 5,6-dimethyl-5,6,7,8-tetrahydropterin or its hydrochloride with methyl iodide takes place only in the presence of an excess of sodium hydroxide. No methylation occurred when a methanolic solution of the dihydrochloride was boiled with an excess of methyl iodide
in the absence of sodium hydroxide, whereas 1,3,5,6-
tetramethyl-5,6,7,8-tetrahydropterinium chloride was
obtained when the neutral molecule of the 5,6-dimethyl
derivative and one molar excess of sodium hydroxide
was treated in the same way (see Section 4.3.1).

The three N-methyl signals of 1,3,5,6-tetramethyl-
5,6,7,8-tetrahydropterinium chloride in the p.m.r.
spectrum (Figure 4.3.b) were tentatively assigned to
CH$_3$(1), CH$_3$(3) and CH$_3$(5), respectively, in increasing
upfield shifts, because the chemical shift of the first
and the second signals were very close to those of
1,3,6-trimethyl-5,6,7,8-tetrahydropterinium chloride
(see Figure 4.3.a). In order to assign the highest
field signal to CH$_3$(5) conclusively, the 5,6-dimethyl-
pterin (4.25) was methylated as before but with excess
of trideuteromethyl iodide instead of methyl iodide.
The p.m.r. spectrum of the product (Figure 4.3.c),
indicated that the 5-methyl group was exchanged, and
that it was transferred equally to N(1) and N(3).
The experiment was repeated because this pattern of
signals was not expected since a trideuteromethyl group
should not give signals in this region of the spectrum.
The product, however, was identical with the one observed
in the first experiment. Further treatment of this
product with CD$_3$I/NaOH or CH$_3$I/NaOH did not alter its
composition (i.e. the spectrum in Figure 4.3.c was
unaltered). This indicated that methyl group exchange
in the tetramethyl derivative did not occur. The
possibility that trideuteromethyl iodide reacted with
Figure 4.3

P.m.r. spectra (methyl signals only) in 0.5N-DCl/D₂O at 60 MHz
methanol in the presence of alkali to give methyl iodide and trideuteromethanol, and that the methyl iodide formed would cause some methylation was excluded because a tetramethyl derivative with a spectrum similar to the one in Figure 4.3.c was obtained when 5,6-dimethyl-5,6,7,8-tetrahydropterin was boiled with CD$_3$I/NaOH in ethanol as solvent. Also the p.m.r. spectra of a solution of trideuteromethyl iodide and sodium hydroxide in methanol had no methyl iodide signal on standing for a long period, or on boiling. The data indicated that alkylation occurred on N(5) to yield the quaternary salt 5-trideuteromethyl-5,6-dimethyl-5,6,7,8-tetrahydropterinium cation (4.26) which is a stronger alkylating agent towards itself than is trideuteromethyl iodide, and can transfer either the methyl group or the trideuteromethyl group to N(1) and N(3).

More insight into the mechanism was obtained by alkylation 3,5,6-trimethyl-5,6,7,8-tetrahydropterin with trideuteromethyl iodide in the presence of sodium hydroxide. The pattern of signals obtained from the product (see Figure 4.3.d) was not as predicted. It is reasonable to assume that the methyl group on N(3) is not displaced and that the highest field N-methyl signal is not from N(5) as suggested above. This signal must be assigned to CH$_3$(3). The spectrum in Figure 4.3.c can now be explained by the sequence 4.25 → 4.27 → 4.28 → 4.29a + 4.29b. Some of the salt 1,3-bis(trideuteromethyl)-5,6-dimethyl-5,6,7,8-tetrahydropterinium chloride (4.29a) may also be formed by direct alkylation of 3-trideuteromethyl-5,6-dimethyl-5,6,7,8-tetrahydropterin.
The first product was the alkylation of 1,5,6,7,8-pentamethyl-5,6,7,8-tetrahydropterin with trimethyl iodide in the presence of sodium hydroxide. 1,5,6,7,8-pentamethyl-5,6,7,8-tetrahydropterin, obtained by reduction of 1,5,6,7,8-pentamethyl-5,6,7,8-tetrahydropterin (4.29a) and (4.29b) forms anhydro-5,6,7,8-tetrahydropterin which showed that the spectrum of this compound had expected changes, that the reduction of the 8-iodo group has occurred. In this case, the 1,5,6,7,8-pentamethyl-1,5,6,7,8-pentamethyl-5,6,7,8-tetrahydropterin is not a good an alkylation agent towards itself as 3,5,6,8-tetrahydropteridinyl is 168
(4.27) with trideuteromethyl iodide. It is not possible to say whether a secondary isotope effect is operating because of the inherent errors in measuring signal heights. It was found, however, that when two methyl groups are present in the pyrimidine ring (which thus has a positive charge), exchange of the methyl group on N(5) does not occur because 1,3,5,6-tetramethyl-5,6,7,8-tetrahydropterinium chloride hydrochloride is unchanged when boiled with trideuteromethyl iodide in methanol containing one molar excess of sodium hydroxide (see above).

The final test was the alkylation of 1,5,6-trimethyl-5,6,7,8-tetrahydropterin with trideuteromethyl iodide in the presence of sodium hydroxide. 1,5,6-Trimethyl-5,6,7,8-tetrahydropterin had been prepared by Armarego by reduction of 5-formyl-1,6-dimethyl-5,6,7,8-tetrahydropterin, obtained by direct formylation of 1,6-dimethyl-5,6,7,8-tetrahydropterin with acetic formic anhydride. The product after the trideuteromethylation of 1,5,6-trimethyl-5,6,7,8-tetrahydropterin had a p.m.r. spectrum (Figure 4.3.e) which showed that the signal from the CH₃(1) was unchanged, that much exchange of the CH₃(5) signal had occurred, and that a trideuteromethyl group had been placed on N(3). It suggests that in this case, the 5-trideuteromethyl-1,5,6-trimethyl-5,6,7,8-tetrahydropterinium cation (4.30) is not as good an alkylating agent towards itself as 3,5-bis(trideuteromethyl)-5,6-dimethyl-5,6,7,8-tetrahydropterinium cation towards itself. Transfer of the methyl group from N(5) to N(1)
appears much more effective than from N(5) to N(3). This may be a reflection of the difference between the nucleophilic nature of N(1) and N(3), and their steric environments.

The three N-methyl resonances in the p.m.r. spectrum of 1,3,5,6-tetramethyl-5,6,7,8-tetrahydropterinium chloride (Figure 4.3.b) can now be assigned from the combined data obtained from the p.m.r. spectra shown in Figures 4.3.c-e. The signal from CH$_3$(5) is at lowest field, this is followed by the CH$_3$(1) signal, and the CH$_3$(3) signal is at the highest field; which is different from the tentative assignment (see p.164).

4.4 Stability of 5,6,7,8-tetrahydropterins

The aerial oxidation of 6-methyl-5,6,7,8-tetrahydropterin and a few methylated derivatives was examined in the present work in order to find out to what extent methylation had stabilized the molecule. Changes in ultraviolet absorption with time were recorded for a period longer than three times the half-lives ($t_{1/2}$) of the respective compounds. Rate constants and $t_{1/2}$ values at 20$^\circ$ were evaluated by Guggenheim's method using a computer analysis. The values are listed in Table 4.1. When air was bubbled through the solutions, there was an increase in rate of 10-20%.

In contrast to the mono- and di-methyl derivatives of 5,6,7,8-tetrahydropterin, it can be seen from Table 4.1 that 1,3,6-trimethyl- and 1,3,5,6-tetramethyl-5,6,7,8-tetrahydropterinium chlorides and 1,3,6-trimethyl-
Table 4.1  Stability of methylated 5,6,7,8-tetrahydropterins in various buffers at 20°

<table>
<thead>
<tr>
<th>Tetrahydropterin</th>
<th>pH 2</th>
<th>pH 8</th>
<th>pH 10</th>
<th>pH 12</th>
</tr>
</thead>
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<tr>
<td></td>
<td>t₁/₂ (h)</td>
<td>rate (h⁻¹)</td>
<td>λ anal (nm)</td>
<td>t₁/₂ (h)</td>
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<td>6-Me</td>
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<td>1,6-diMe*</td>
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<td></td>
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<td>2',6-diMe₅</td>
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<td>.20x10⁻²</td>
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<td></td>
</tr>
<tr>
<td>5,6-diMe</td>
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<td>too slow to measure</td>
<td></td>
<td>27h</td>
</tr>
<tr>
<td>6,7-diMe(cis) (cis + trans)</td>
<td>5.2h</td>
<td>.13h⁻¹</td>
<td>266</td>
<td>42</td>
</tr>
<tr>
<td>6,8-diMe*</td>
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<td></td>
<td>22</td>
</tr>
<tr>
<td>1,3,6-triMe⁺ (Cl⁻, HCl)*</td>
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<td></td>
</tr>
<tr>
<td>1,2',3,6-tetraMe⁺ (Cl⁻, HCl)₅</td>
<td>too slow to measure</td>
<td>too slow to measure</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Minutes unless otherwise stated.
b This compound is 6-methyl-2-methylamino-4(3H)-oxo-5,6,7,8-tetrahydropteridine.
c This compound is 1,3,6-trimethyl-2-methylamino-4(3H)-oxo-5,6,7,8-tetrahydropteridinium chloride hydrochloride.

* Kindly supplied by Dr W.L.F.Armarego.
### Table 4.2

Stability of methylated 5,6,7,8-tetrahydropterins at 20° in various buffers containing copper and glycine

<table>
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<tr>
<th>Tetrahydropterin</th>
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<th></th>
<th>pH 12</th>
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<td>$\lambda_{anal}$ (nm)</td>
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<td>299</td>
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<tr>
<td>1,6-diMe*</td>
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<td>.70x10$^{-2}$</td>
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<tr>
<td>2',6-diMe*</td>
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$^a$ 1,3,6-triMe$^+(\text{Cl}^-, \text{HCl})^*$

$^b$ 1,2',3,6-tetraMe$^+(\text{Cl}^-, \text{HCl})^*$ too slow to measure at pH 8 and 12

$^c$ 1,3,5,6-tetraMe$^+(\text{Cl}^-, \text{HCl})$

$a$ 10$^{-4}$ M Cu$^{2+}$ and 3x10$^{-4}$ M glycine.

$b$ .5x10$^{-4}$ M Cu$^{2+}$ and 1.5x10$^{-4}$ M glycine.

$c$ 2x10$^{-4}$ M Cu$^{2+}$ and 6x10$^{-4}$ M glycine.

$d$ $T_{1/2}$ ~10 min for initial reaction.

* Kindly supplied by Dr W.L.F. Armarego.
2-methylamino-4(3H)-oxo-5,6,7,8-tetrahydropteridinium chloride show no changes in ultraviolet absorption on standing in aqueous buffers of pH 2 to 12.

Trace metals, such as ferric and cupric ions, are generally known to accelerate the oxidation process, as mentioned in Section 4.2. The measured oxidation rates (rate constants and $t_{1/2}$ values) at $20^\circ$ in the presence of Cu$^{2+}$ ($10^{-4}$M) and glycine ($3\times10^{-4}$M) are shown in Table 4.2 (to be compared with Table 4.1). The glycine was added as a chelating agent in order to prevent precipitation of copper as hydroxide in basic solution. Generally, copper ion catalyze the oxidation, except for 6,7- and 6,8-dimethyl-5,6,7,8-tetrahydropterin in which the rate decreased in the presence of copper ions, and decreased further when the copper ion concentration was increased. A fast initial rate was observed especially in the case of 6-methyl- and 1,6-dimethyl-5,6,7,8-tetrahydropterin during the first minute. (The initial parts of the u.v. absorption curves were therefore excluded in the computer analysis.) However, this phenomenon was not investigated further because it was considered of minor importance within the scope of the present problems.

In conclusion, it has been shown that the methylation of 6-methyl-5,6,7,8-tetrahydropterin has stabilized the molecule considerably towards aerial oxidation.

4.5 Mechanism of N(5)-demethylation

Removal of the N(5)-methyl group in 1,3,5,6-tetramethyl-5,6,7,8-tetrahydropteridinium chloride by elution with 3N-aqueous ammonia from a Dowex 50W column
can also be effected by heating the salt with N-aqueous sodium hydroxide or aqueous ammonia but less efficiently. The salt, on the other hand, is very stable in the presence of strong acid (no change could be observed in the ultraviolet spectrum over a period of three days). Aqueous ammonia (3N, with and without Dowex 50W), aqueous sodium hydroxide or 3N-hydrochloric acid do not demethylate 5,6-dimethyl- or 3,5,6-trimethyl-5,6,7,8-tetrahydropterin. No oxidation of the pterin is involved in the demethylation of 1,3,5,6-tetramethyl-5,6,7,8-tetrahydropterinium chloride because it proceeds equally effectively whether mercaptoethanol is present or not in the aqueous ammonia solution used in the chromatographic purification. Clearly the pyrimidine ring must be positively charged for the removal of the 5-methyl group. The strong electron withdrawing property of the positively charged pyrimidine ring must make N(5) electron deficient, increase the polarization CH$_3$$^{δ+}$-N(5)$^{δ-}$, and allow nucleophilic attack on CH$_3$(5) (see 4.31 + 4.32). This is revealed in the p.m.r. spectrum of the tetramethyl salt (4.17)(see Figure 4.3.b). The N-methyl signal at lowest field ($δ$ 4.38 p.p.m.) is from CH$_3$(5) which is the most deshielded methyl group, i.e. more deshielded than the 1- and 3-methyl group which are on a positively charged heteroaromatic nucleus. The intermediate (4.32) is stabilized by its zwitterionic nature, but is then protonated to give 1,3,6-trimethyl-5,6,7,8-tetrahydropterinium chloride.
The mechanism by which 5-methyl-5,6,7,8-tetrahydrofolate acid (the first intermediate in many biological methylations) transfers its methyl group to homocysteine (4.31) is not clearly understood. The modification of N(5) in the pterin ring is involved in this process, and in connection with this work it has been shown that the 2-methyl group in 5-methyl-5,6,7,8-tetrahydrofolate acid is transferred to deoxyadenosylcobalamin and then to homocysteine, the enzyme must bind very strongly to the nitrogen atoms in order to assist in the release of CH₃CNH₂CNH₂ and methionine takes place in the 2,3,6-trimethylfolic acid transferase enzyme system.

The exchange of CH₃CNH₂CNH₂ in 5-methyl-5,6,7,8-tetrahydropterin allows an alternative mechanism for the methyl group transfer of 5-methyl-5,6,7,8-tetrahydrofolic acid. In this mechanism N(5) may be methylated by some biological electrophilic centre on the enzyme, which would make the quaternized folic acid (at N(5)) a powerful alkylating agent that can transfer the 5-methyl group to a receptor molecule. The 5-unsubstituted coenzyme (5,6,7,8-tetrahydrofolate acid) can then be regenerated by the citrulline mechanism discussed above.
The mechanism by which 5-methyl-5,6,7,8-tetrahydrofolic acid (the first intermediate in many biological methylations), transfers its 5-methyl group to homocysteine (4.33) to make methionine (4.34) is not clearly understood. The demethylation of N(5) in the pterins discussed by Armarego and in connection with this work suggests that if the 5-methyl group in 5-methyl-5,6,7,8-tetrahydrofolic acid is to be transferred to deoxyadenosylcobalamin and then to homocysteine, the enzyme must bind very strongly to the nitrogen atoms in the pyrimidine ring to assist in the release of CH₃(5) to deoxyadenosylcobalamin. It has been shown that transfer of radioactivity from 5-methyl[¹⁴C]-tetrahydrofolic acid to methylcobalamin and methionine takes place in the E. coli B methyltransferase enzyme system.

Exchange of CH₃(5) in 5,6-dimethyl-, 3,5,6-trimethyl- and 1,5,6-trimethyl-5,6,7,8-tetrahydropterin allows an alternative mechanism to be proposed for the enzymatic transfer of CH₃(5) in 5-methyl-5,6,7,8-tetrahydrofolic acid. In this mechanism N(5) may be methylated by some biological electrophile, or an electrophilic centre on the enzyme, which would make the quaternized folate [at N(5)] a powerful alkylating agent that can transfer the 5-methyl group to a receptor molecule. The 5-unsubstituted coenzyme (5,6,7,8-tetrahydrofolic acid) can then be regenerated by the cleavage mechanism discussed above.
4.6 Experimental

Analytical samples were dried at 100° and 1 mmHg for 3 h unless otherwise stated. U.v. spectra of the compounds in buffered solutions were measured on a Unicam SP800 spectrophotometer, and the maxima were measured on a Unicam SP500 manual spectrophotometer. (++, +, 0 and − refer to di-cation, mono-cation, neutral species and anion respectively). P.m.r. spectra (chemical shifts in p.p.m., and J values in Hz) were measured on a Varian T60A spectrometer, and all solutions were in 0.5N-DCl/D₂O (10-25 mg in 0.25 ml) with tetramethylsilane (δ = 0 p.p.m.) as external standard in an inserted tube. All the spectra were locked on the external standard and the resolution was maximized prior to each run. Evaporations were carried out in a Büchi evaporator below 30° and at ca. 18 mmHg, with the receiver immersed in an ice bath. ¹³C n.m.r. spectra (67.89 MHz) were run in 0.5N-DCl/D₂O with dioxane as internal standard (δ 67.6 p.p.m.) by Dr A.J. Jones and staff at the National NMR Centre, Canberra, A.C.T. For further details, see Part 2, Experimental (Section 2.7, p.91).

General method for the preparation of 5,6,7,8-tetrahydropteridine hydrochlorides.

A general method for catalytic reduction of pteridines is described in Part 2, Experimental (Section 2.7, p.115).

5,6-Dimethyl-5,6,7,8-tetrahydropterin hydrochloride (see also later). This was prepared by the method of Whiteley, Drais and Huennekens ²⁰⁴ (using HCHO and KBH₄) in 48% yield (lit., ²⁰⁴ 52%); m.p. 228° (decomp; Whiteley et al. do not give a melting point); p.m.r. and u.v. spectra were identical with the
reported; \( \nu_{\text{max.}} \) 3250 (NH), 2900 (CH str), 2720, 1720 (CO), 1660 (NH) and 1460 (Found: C, 34.2; H, 5.8; N, 24.9; Cl, 25.3. Calc. for \( \text{C}_8\text{H}_{13}\text{N}_5\text{O}, 2.0 \text{HCl}, 0.75 \text{H}_2\text{O}: \) C, 34.1; H, 5.9; N, 24.9; Cl, 25.2%).

5-Formyl-6-methyl-5,6,7,8-tetrahydropterin. 6-Methyl-5,6,7,8-tetrahydropterin hydrochloride (2 g of 1.7 hydrochloride) in 98-100% formic acid (80 ml) under dry nitrogen was treated gradually with acetic anhydride (20 ml) (effervescence on standing) below 25°. After standing in the dark at 20° for 24 h a further amount of acetic anhydride (20 ml) was added to the mixture which was then kept at 20° for another 24 h. A third quantity of acetic acid (20 ml) was added, and after keeping at 20° for 24 h the solution was evaporated to dryness and the excess acid and anhydride were chased with chloroform. The crude derivative (80-90%) was satisfactory for further reduction to 5,6-dimethyl-5,6,7,8-tetrahydropterin (see later). To obtain an analytical sample, the residue (500 mg) was dissolved in water (1 ml) and saturated aqueous sodium carbonate was added dropwise until effervescence ceased (pH 6-7). A solid separated only after persistent scratching with a glass rod at 0°, and the crystals were collected, washed with a few drops of water and recrystallized once from a very small volume of water. It is important to use the volumes indicated because the derivative is appreciably soluble in the solution. The 5-formyl-6-methyl-5,6,7,8-tetrahydropterin had m.p. 289-289.5° (decomp.) [lit., 206° 282-286° (decomp.) for the half hydrate prepared by a slightly different method]; \( \delta(\text{CF}_3\text{COOD}) \) 9.12 and 8.47 (two s, 3:1, CHO), 5.22 [1H, br s, H(6)], 3.67 [2H, d, two H(7)], 1.45 and 1.30 [two d, 1:3, J 6 and 7, CH\(_3\)(6)]; \( \nu_{\text{max.}} \) 3350 (br, NH), 2950 (br), 2750 (br), 1650 (br, amide), 1585 (amide) and 1563 (Found,
after drying at 100° for 1 h: C, 42.3; H, 5.9; N, 30.7.

6-Methyl-5,6,7,8-tetrahydropterin hydrochloride was dissolved in formic acid and boiled for 30 minutes under nitrogen. No acetic anhydride was added. This treatment caused 40% formylation.

Stirring at room temperature with acetic formic anhydride for 3 days under nitrogen gave 29% of the formyl derivative of tetrahydropterin. Heating at 80° for 6 h increased the yield only slightly (by 4%).

5-Formyl-6-methyl-5,6,7,8-tetrahydropterin was obtained in 82% yield by reaction with 90% formic acid (1.1 ml; 29 mol. equiv.) and formaldehyde (46 µl 36%; 1.2 mol. equiv.) at 80° for 9 h under nitrogen.

5,6-Dimethyl-5,6,7,8-tetrahydropterin hydrochloride.—This salt was prepared in 80-90% yield by catalytic reduction of 5-formyl-6-methyl-5,6,7,8-tetrahydropterin (crude) by the general method described above, except that reduction required 10-24 h, and was monitored by examination of the p.m.r. spectrum of an aliquot. Increased hydrogen pressure (100 atm) caused formation of platinum salts which are difficult to remove. The product had identical spectra with those of the dimethylpterin hydrochloride prepared by the method of Whiteley, Drais and Huennekens, but the analysis (with respect to HCl and H2O content) was slightly different. (Found: C, 34.6; H, 5.8; N, 25.2; Cl, 24.4. C8H13N5O, 1.9 HCl, 0.75 H2O requires C, 34.6; H, 5.9; N, 25.2; Cl, 24.2%). It had m.p. 228° (decomp; lit.,205 238-242°).
δ(CF<sub>3</sub>COOD) 3.59 [3H, s, CH<sub>3</sub>(5)] and 1.88 [3H, d, J 5.8, CH<sub>3</sub>(6)]; λ<sub>max</sub> (log ε) at H<sub>2</sub>O -1 (+) 264 (4.23), at pH 3.6 (+) 219 (4.10) and 265 (4.10), at pH 8.5 (0) 286 (4.04) and at pH 13 (-) 279 (3.94).

1,3,6,7-Tetramethyl-5,6,7,8-tetrahydropterinium chloride hydrochloride.—6,7-Dimethyl-5,6,7,8-tetrahydropterin hydrochloride (200 mg) was dissolved in methyl p-toluenesulphonate (5 g; 36 mol. equiv.) and heated at 130° for 1 h. Ether was added to the cold mixture and 1,3,6,7-tetramethyl-5,6,7,8-tetrahydropterinium p-toluenesulphonate (260 mg; 87%) was collected. This salt was purified through a Dowex 50W column in 43% yield (see preparation of 1,3,5,6-tetramethyl for methylation of 5,6,7,8-tetrahydropterin hydrochlorides). The tetramethyltetrahydropterin had m.p. >220° (effervescence); δ 4.28 [3H, s, CH<sub>3</sub>(1)], 4.12 [3H, s, CH<sub>3</sub>(3)] and 1.93 [6H, d, CH<sub>3</sub>(6), CH<sub>3</sub>(7)]; ν<sub>max</sub> 220 (1.00) and 264 (0.34) at pH 2, 7 and 12 (Found: C, 37.2; H, 6.4; Cl, 27.4; N, 21.3. C<sub>10</sub>H<sub>18</sub>CIN<sub>5</sub>O, 1.5 HCl, 0.5 H<sub>2</sub>O requires C, 37.1; H, 6.4; Cl, 27.4; N, 21.6%).

An attempt to prepare 5-benzyl-6-methyl-5,6,7,8-tetrahydropterin as indicated by Matsuura and Sugimoto<sup>191,207,208</sup> was unsuccessful.—The hydrochloride was dissolved in ethanol containing a little dry HCl (∼ 1N) and treated with benzaldehyde. The reaction mixture was stirred overnight at room temperature and reduced catalytically (H<sub>2</sub>/PtO<sub>2</sub>). However, only starting material was isolated.
1,3,5,6-Tetramethyl-5,6,7,8-tetrahydropterinium chloride hydrochloride.-5,6-Dimethyl-5,6,7,8-tetrahydropterin hydrochloride (268 mg) was dissolved in methanol (30 ml). 2N-Sodium hydroxide (1.5 ml; 3 mol. equiv.) was added followed by methyl iodide (0.62 ml; 10 mol. equiv.), and the reaction mixture was refluxed under nitrogen for 3 h. Evaporation of the final solution, which had become neutral, left a white-yellow residue. The p.m.r. spectrum of the solid showed that it was a tetramethyl derivative of 5,6,7,8-tetrahydropterin; δ 4.38 [3H, s, CH₃(5)], 4.23 [3H, s, CH₃(1)], 4.05 [3H, s, CH₃(3)] and 2.25 [3H, d, CH₃(6)] (see Figure 4.3.b). A second treatment did not methylate the product further. Sodium iodide was removed by placing the residue (400 mg) on a Dowex 50W column (60 ml, H⁺ form, 50-100 mesh x 4) and washing with water until the acidic eluate (aqueous HI) became neutral. The column was then washed with N-hydrochloric acid [to remove sodium ions (no pterin came through at this stage)] and eluted with 3N-hydrochloric acid until the eluate gave no u.v. spectrum of the tetrahydropterin. The combined eluates (300 ml) were evaporated and the residue (200 mg; 68%) was re-reduced (H₂/PtO₂/TFA) and recrystallized from methanol/ether. It had m.p. 210° (decomp.); δ 4.22, 4.07, 3.92 and 2.09 (J 6.2 Hz); 13C n.m.r. δ (assignment, multiplicity, and J in proton-coupled spectrum) 154.9 [C(4), s], 150.8 [C(2), s], 145.8 [C(8a), s], 98.5 [C(4a), s], 68.6 [C(6), d, J 150], 54.9 [CH₃(3), q, J 150], 50.5 [CH₃(1), q, J 150], 41.4 [C(7), t, J 150], 29.6 [CH₃(5), q, J 150] and 13.4 [CH₃(6), q, J 130] (see Figure 4.1.a and 4.1.b); ν max. 1670 (C=O), 1630, 1590, 1545 and 1530; λ max. (log ε) at H₂O -1 (++) 264 (4.18) and at pH 3.4-12.7 (+) 220 (4.43) and 264 (4.04) (Found: C, 37.3; H, 6.2; Cl, 27.2; N, 21.4. C₁₀H₁₈ClN₁O₅ requires C, 37.2; H, 6.2; Cl, 27.4; N, 21.7%).
General method for trideuteromethylation of 5,6,7,8-tetrahydropterin hydrochlorides

5,6,7,8-Tetrahydropterin hydrochlorides were trideuteromethylated by substituting methyl iodide with trideuteromethyl iodide in the previously described method for methylation (see the methylation of 5,6-dimethyl-5,6,7,8-tetrahydropterin hydrochloride).

The results of methylation and trideuteromethylation of 5,6-dimethyl-5,6,7,8-tetrahydropterin hydrochloride as described above are summarized in Table 4.3.

The p.m.r. spectra of the deuterated tetramethylpterinium salts formed by trideuteromethylation of 5,6-dimethyl-, 3,5,6-trimethyl- and 1,5,6-trimethyl- 5,6,7,8-tetrahydropterin hydrochlorides, measured without the ion exchange purification, are shown in Figure 4.3.c, 4.3.d and 4.3.e, respectively.

In an attempt to obtain a 5,6-dihydropterin, a reductive alkylation of 2,4,5-triaminopyrimidin-6(1H)-one was tried. Sugimoto and Matsuura have shown that polyamino-pyrimidines can be alkylated exclusively at the 5-position by reduction of the Schiff base over platinum catalyst. The Schiff base was formed in the present work by treating the pyrimidine with pyruvaldehyde dimethyl acetal. However, the final ring closure could not be achieved by (a) heating in 3N-aqueous hydrochloric acid for 30 min, (b) heating in conc. hydrochloric acid for 2 h, and (c) heating in methanol containing dry ammonia in a sealed tube for 3½ h.
Table 4.3

Methylation and trideuteromethylation of 5,6-dimethyl-5,6,7,8-tetrahydropterin hydrochloride

\[
\begin{align*}
\text{THP-1} & \xrightarrow{\text{CH}_3} \quad R(5)^* \quad R(1)^* \quad R(3)^* \quad \text{CH}_3(6)^* \\
\text{THP-1} & \xrightarrow{\text{CD}_3} \quad \text{THP-2} \quad 1.5 \quad 1.5 \quad 0 \quad 3 \\
\text{THP-1} & \xrightarrow{\text{CD}_3} \quad \text{THP-2} \quad 1.4 \quad 1.4 \quad 0 \quad 3 \\
\text{THP-2} & \xrightarrow{\text{CH}_3} \quad 1.6 \quad 1.6 \quad 0 \quad 3 \\
\text{THP-2} & \xrightarrow{\text{CH}_3} \quad 1.5 \quad 1.5 \quad 0 \quad 3 \\
\text{THP-1} & \xrightarrow{\text{CH}_3} \quad \text{EtOH} \quad 3 \quad 3 \quad 3 \quad 3 \\
\text{THP-1} & \xrightarrow{\text{CD}_3} \quad \text{EtOH} \quad 1.5 \quad 1.6 \quad 0 \quad 3 
\end{align*}
\]

*Relative intensity of the methyl signal in the p.m.r. spectrum of the product THP-X

PART II
5.1 Introduction

In Part 4 it was shown that the stereoisomers of 6-methyl-5,6,7,8-tetrahydropterin could be stabilized towards aerial oxidation by methylation. The next step on the way to the elucidation of the absolute configuration of the stable enantiomers of 1,3,5,6-tetramethyl-5,6,7,8-tetrahydropterinium chloride would be to find a way of degrading the molecule. As mentioned briefly in Part 4, Mager, among others, found that a spirohydantoin is formed from aerial oxidation of, e.g., 1,3,5-trimethyl-5,6,7,8-tetrahydro-1umazine. This and other experiments suggest that the piperazine ring is more stable than the pyrimidine ring. It should therefore be possible to degrade the tetrahydropterin to a piperazine via a lumazine. The suggested pathway is schematically presented in Scheme 5.1. Treatment of 1,3,5,6-tetramethyl-5,6,7,8-tetrahydropterinium chloride (5.1) with nitrous acid is likely to give the corresponding lumazine (5.2). The pyrimidine ring of the latter can probably be cleaved by sodium hydroxide to yield a methylaminocarboxylic acid (5.3) which is unstable and will decarboxylate to the amide (5.4).
Scheme 5.1
Further hydrolysis will yield an $\beta$-hydroxycarboxylic acid (5.5) which should be unstable and decarboxylate to 1,2-dimethyl-5-oxopiperazine (5.6). Methylation of this ketopiperazine at N(4) followed by reduction of the carbonyl group will provide the key compound (5.7) for correlating the absolute configuration at the asymmetric centre.

In this way it should be possible to establish the absolute configuration at C(6) in the parent tetrahydropterin by comparing the chiroptical properties of the degradation product with those of 1,2,4-trimethylpiperazine of known absolute configuration at C(2).

The present Part describes the preparation and chiroptical properties of one enantiomer of 3-methyl-2,5-diketopiperazine, 2-methylpiperazine and the key intermediate 1,2,4-trimethylpiperazine hydrochloride.

5.2 Discussion

5.2.1 Preparation and chiroptical properties of
2S-(−)-2-methylpiperazine hydrochloride

The total synthesis of 2S-(−)-2-methylpiperazine (5.13) is shown in Scheme 5.2.

The formation of the unprotected dipeptide, glycyl-$S$-alanine methyl ester hydrochloride (5.11), was achieved by applying the mixed carbonic anhydride method described by Anderson, Zimmerman and Callahan using ethyl chloroformate (see Eqns. 5.8 $\rightarrow$ 5.9 $\rightarrow$ 5.10 $\rightarrow$ 5.11).
Scheme 5.2 Preparation of 2S-(-)-2-methylpiperazine
The scope and limitations of the use of alkyl chloroformates as peptide-forming reagents have been discussed in a review article by Albertson. The most serious limitation is the danger of racemization when acylpeptides are used as coupling components. However, experience has shown that acylamino acids can be used without racemization when e.g. a carbobenzoxy group is used as protecting group. Carbobenzoxyglycine (5.8) was prepared from glycine and benzyl chloroformate following the directions of Carter, Frank and Johnston. The carbobenzoxy blocking group was removed by hydrogenolysis in methanol containing a little dry hydrogen chloride over 50% Pd/C (5.10 → 5.11) as described by Bláha. It could also be removed by treatment with 35% hydrogen bromide in acetic acid solution. The product, glycy1-S-alanine methyl ester hydrochloride, had a specific rotation of $\alpha = -50^0$ in water at 589 nm and 20°. The cyclization (5.11 → 5.12) was achieved by treating the peptide dissolved in methanol with methanolic ammonia. The 3S-(−)-3-methyl-2,5-diketopiperazine obtained had $[\alpha]_{589}^{20} = -3.6^0$ in water. Fischer and Abderhalden, who obtained the dipeptide by hydrolysis of silk fibroins, found a specific rotation of $-3.9^0$ under the same conditions as above. The o.r.d. curves of 3S-(−)-3-methyl-2,5-diketopiperazine in water and ethanol are shown in Figure 5.1 and compared well with those reported in the literature. The melting point and rotation were misquoted by Davis and Abu Khaled.
Figure 5.1

$\alpha$-r.d. of $3S-( - )$-3-methyl-2,5-diketopiperazine

- in water
- in ethanol

$\lambda$ 220 240 260 280 300 320 340 360 380 400 nm
The reduction of 3\(S\)-\((-\))-3-methyl-2,5-diketopiperazine to 2\(S\)-\((-\))-2-methylpiperazine (5.12 ~ 5.13) could not be achieved with lithium aluminium hydride, not even after boiling in 'glyme' under reflux for six days with a large excess of reducing agent. Catalytic reduction (H\(_2\)/PtO\(_2\)/TFA) also failed. Reduction was finally attained by using 'Vitride' [sodium dihydro-bis(2-methoxyethoxy)aluminate], which resulted in the formation of the methyl derivative in 37\% yield. The product, as a hydrochloride, had [\(\alpha\)]\(_{435}^{20}\) -3.0\(^\circ\) in 2N-aqueous hydrochloric acid (see also Experimental).

5.2.2 Preparation and chiroptical properties of 2\(S\)-\((+\))-1,2,4-trimethylpiperazine hydrochloride

The synthesis of 2\(S\)-\((+\))-1,2,4-trimethylpiperazine hydrochloride is based upon the method described by Weipert\(^{216}\) for the preparation of the racemic compound. Methylation of 2\(S\)-\((-\))-2-methylpiperazine was achieved with a mixture of formaldehyde and sodium carbonate in excess, and the piperazine was obtained pure in 52\% yield \(\text{via}\) the picrate. Lower yields (~10\%) were obtained when the trimethyl derivative (racemic) was prepared by a 'Vitride'-reduction of the 1,4-diformyl derivative which had been made from 2-methylpiperazine by treatment with formic acid and acetic anhydride (as for the formylation of 6-methyl-5,6,7,8-tetrahydropterin).
The difficulties encountered in the reduction of the formylpiperazine are significant and reflect on the difference in properties of the formylpiperazine compared with the related 5-formyl-6-methyl-5,6,7,8-tetrahydropterin. 2S-(+)-1,2,4-trimethylpiperazine hydrochloride had a specific rotation of +3.8° at 435 nm and 20° in 2N-aqueous hydrochloric acid (see Experimental for further data).
5.3 Experimental

The experimental data included in this Section were obtained under the same conditions as described in the introduction to the Experimental Section in Part 2, p.91, Part 3, p.130 and Part 4, p. 177.

**Carbobenzoxyglycine.**—This was prepared from glycine and benzyl chloroformate by the method described by Carter, Frank and Johnston\(^{211}\) in 71\% yield. It had m.p. 118-120\(^\circ\), and 119.5-120.5\(^\circ\) after recrystallization from chloroform-cyclohexane (lit.,\(^{211}\) m.p. 119-120\(^\circ\); yield 86-91\%; \(\delta(DMSO)\) 7.87 (5H, s, \(C_6H_5\)), 5.55 (2H, s, Ph-CH\(_2\)) and 4.08 (2H, d, CH\(_2\)); the doublet from the methylene group became a singlet on addition of a few drops of D\(_2\)O because of H/D exchange at the amide nitrogen atom. \(\nu_{\text{max}}\) 3355 (NH), 1730, 1710 (CO), 1695, 1680, 1260 (NH) and 985.

**Carbobenzoxyglycyl-S-alanine methyl ester.**—A solution of carboxbenzoylglycine (1.046 g) in tetrahydrofuran (25 ml; distilled from calcium hydride) was cooled to -15\(^\circ\) with stirring. Triethylamine (0.70 ml; 1 mol. equiv.) was added followed by freshly distilled ethyl chloroformate (0.47 ml; 1 mol. equiv.) which precipitated triethylamine hydrochloride. After this mixture had been stirred for 12 min, S-alanine methyl ester [from the hydrochloride (0.52 g; 1 mol. equiv.) by addition of an equivalent amount of triethylamine (0.70 ml)] suspended in tetrahydrofuran (5 ml) was added. Stirring was continued for \(\sim\) 1 min at -15\(^\circ\) whereafter the reaction mixture was allowed to warm to room temperature. The solvent was evaporated and ethyl acetate (75 ml)
and 5% aqueous sodium carbonate (25 ml) were added. The mixture was shaken and allowed to separate. The ethyl acetate fraction was collected, washed with water (25 ml), N-hydrochloric acid (25 ml) and water (25 ml). Drying and evaporation of the solvent left carbobenzoxyglycyl-S-alanine methyl ester as an oil (0.956 g; 65% (83% in synthesis using racemic alanine)); \( \delta(\text{CDCl}_3) \) 7.43 [5H, s, \( \text{C}_6\text{H}_5 \)], 5.18 [2H, s, Ph-CH\(_2\)H], 4.63 [1H, q, CH], 3.93 [2H, d, CH\(_2\) (glycyl)], 3.75 [3H, s, OCH\(_3\)] and 1.37 [3H, d, CH\(_3\) (alanine)].

**Glycyl-S-alanine methyl ester hydrochloride.**—The preceding ester (680 mg) in methanol (50 ml) acidified with methanolic hydrogen chloride was hydrogenolyzed by bubbling hydrogen through the solution containing freshly prepared 50% Pd/C (100 mg) for 1½ h. It was found of importance to use as active a catalyst as possible (10% Pd/C was unsatisfactory). The catalyst was removed and the solvent evaporated. The oily residue was dissolved in chloroform (50 ml) and light petroleum (b.p. 40-60°) was added until the mixture became slightly turbid whereupon it was left at -5° for 3 days. The crystals of glycyl-S-alanine methyl ester hydrochloride were collected and dried in a vacuum over KOH and H\(_2\)SO\(_4\) overnight. The hydrochloride (324 mg; 71%) had \([\alpha]_{589}^{20} -50°\) in water (rough estimate; lit. \([\alpha]_{589}^{217} -50°\); the p.m.r. spectrum showed no aromatic protons; \( \delta(\text{CDCl}_3) \) 4.4 [1H, q (almost disappeared in a band envelope), CH], 3.73 [2H, d, CH\(_2\)H], 3.53 [3H, s, OCH\(_3\)] and 1.20 [3H, d, CH\(_3\) (alanine)]; \( \nu \) max. 3480 (NH), 1860, 1710 (CO), 1545, 1460 and 1215; it had no ultraviolet absorption spectrum.
3S-(-)-3-Methyl-2,5-diketopiperazine.-Glycyl-S-alanine methyl ester hydrochloride (394 mg) was dissolved in absolute methanol (10 ml), and the solution was basified with methanolic ammonia (20 ml) and left at room temperature overnight. A white solid precipitated. Saturation of the methanolic solution with ammonia gas did not cause further precipitation (on the contrary, some solid appeared to pass into solution). The solid (240 mg; 93%) was collected and dried, and had m.p. 243-244° (decomp.) and [α] 589$^\circ$ -3.5° and [α] 546$^\circ$ -3.9°. The piperazine sublimed slowly at 250° and ~ 0.5 mmHg in 79% yield. The sublimate had m.p. 246-247° and [α] 589$^\circ$ -3.6° in water (lit., 218 m.p. 240°; lit., 213 [α] 20$^\circ$ -3.9°). See Figure 5.1 and the following for o.r.d. curves and data, respectively. δ(DMSO) 3.93 [1H, q, H(3)], 3.82 [2H, s, 2 H(6)] and 1.29 [3H, d, CH$_3$(3)]; ν max. 3460 (br, NH), 2940, 2900 (CH str), 1690 (CO), 1665, 1465 and 1320 (Found: C, 46.8; H, 6.1; N, 21.7. Calc. for C$_5$H$_8$N$_2$O$_2$: C, 46.9; H, 6.3; N, 21.9%).

2S-(-)-2-Methylpiperazine hydrochloride.—3S-3-Methyl-2,5-diketopiperazine (1.46 g) was dissolved in dry benzene (100 ml) and treated with 'Vitride' (10 ml of 70% in benzene). The reaction mixture was boiled under reflux for 2 days, and left at room temperature overnight after a further amount of 'Vitride' (5 ml) had been added. Excess of 'Vitride' was destroyed by slow addition of saturated aqueous potassium carbonate (vigorous exothermic reaction). The mixture was filtered and the gummy residue extracted thoroughly with benzene. The combined benzene fractions were dried, and the solvent was evaporated. The residue (424 mg; 37%) was dissolved in 4N-hydrochloric acid (10 ml), evaporated and dried in a vacuum desiccator overnight (KOH). The methylpiperazine hydrochloride
### O.r.d. data

**3β-(-)-3-Methyl-2,5-diketopiperazine**

42.16 mg in 2.00 ml H₂O

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*Solution diluted ten times.
0.r.d. data 3S-(−)-3-Methyl-2,5-diketopiperazine

27.34 mg in 25.00 ml EtOH

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* Solution diluted ten times.
was sublimed in 87% yield at 190° and 2 mmHg. The sublimate had m.p. 250-251° (lit., 217 248-249° for the racemate); δ(2N-DCl) 4.1 [7H, multiplet, 3 CH₂ and H(2)] and 2.02 [3H, d, CH₃(2)]; δ(CDC₃) 2.8, 1.57 [2H, s, 2 NH] and 0.98; νmax. 3550, 3480 (NH and H₂O), 2940 br, 2820, 2750 (CH str), 1570 (NH), 1460, 1435 and 1420, but no carbonyl peak (Found: C, 34.65; H, 8.2; N, 15.8. Cl, 40.9. C₅H₁₂N₂, 2 HCl requires C, 34.7; H, 8.2; N, 16.2; Cl, 41.0%).

2S-(-)-2-Methylpiperazine hydrochloride (82.30 mg) in 2.00 N-HCl (2.00 ml) had:

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2S-2-Methylpiperazine picrate.-This was prepared in 87% yield from the preceding piperazine (free base). The picrate had m.p. 278-280° (decomp.; lit., 217 276-278° for the racemate (di(?)picrate)); νmax. 3480 (NH, H₂O), 2980, 2820 (CH str), 1610 (NH) 1575, 1540, 1370 and 1335 (Found: C, 37.45; H, 3.35; N, 20.0. C₅H₁₂N₂, 1.7 picrate requires C, 37.3; H, 3.5; N, 20.3%). The free base was obtained by addition of an excess of 10N-sodium hydroxide and heated to 95° followed by thorough extraction of the cold mixture with chloroform. The free base (70%) was spectroscopically pure (p.m.r.).

1,4-Diformyl-2-methylpiperazine.-Racemic 2-methylpiperazine (3.0 g) was dissolved in formic acid (60 ml) at 0°. Acetic anhydride (30 ml) was added which caused the reaction to boil. After standing overnight at 20° further amounts of acetic anhydride (4 x 10 ml) were added during a period of 9 h. The reaction mixture was left at
room temperature overnight and then evaporated to dryness. The remaining acid and anhydride were chased with chloroform and the residue was distilled at 46-48° and 27 mmHg. The formylpiperazine (3.83 g; 82%) had δ(CDCl₃) 8.17 and 8.03 [2 x 1H, two s, ca. 1:1 CHO], 3.37 [ν7H, multiplet in band envelope, 3 CH₂ and H(2)], 1.32 and 1.20 [2 x 3H, two d, ca 1:1, CH₃(2)]; δ(CD₃COOH) 8.43 and 8.30, 3.75, 1.52 and 1.40; ν max. 3450 (br, NH), 2960, 2900 (CH str), 2720 (w, CHO), 1780, 1770 (CO), 1650, 1450, 1400, 1215 and 1175 (Found: C, 53.7; H, 7.9; N, 17.8. C₇H₁₂N₂O₂ requires C, 53.8; H, 7.7; N, 17.9%).

1,2,4-Trimethylpiperazine.-The preceding 1,4-diformyl-2-methylpiperazine (1.0 g) was dissolved in benzene (50 ml) and treated with 'Vitride' (10 ml of 70% in benzene). The reaction mixture was boiled under reflux for 5 h whereafter the excess of 'Vitride' was destroyed by addition of saturated aqueous potassium carbonate. The mixture was boiled for 30 min and filtered cold. The white gummy residue was extracted thoroughly with tetrahydrofuran. Evaporation of the combined and dried fractions gave an oily residue. Its p.m.r. spectrum showed that only ~ 10% reduction of the formyl groups had occurred.

Hardly any reduction of the formyl derivative (1 g) took place on shaking with hydrogen at 720 mmHg over PtO₂ (250 mg) in trifluoroacetic acid (25 ml) for a week as shown by p.m.r. spectroscopy.

2S-(+)-1,2,4-Trimethylpiperazine hydrochloride.-To a mixture of formaldehyde (1.2 g of 37% aqueous solution; 6.0 mol. equiv.) and sodium carbonate (0.13 g; 0.5 mol. equiv.) was added an aqueous solution of 2S-(−)-2-methylpiperazine [from the dihydrochloride (394 mg) and 2 mol. equiv. of sodium hydroxide]. The reaction mixture was
stirred for 30 min (exothermic reaction) followed by heating at 90-95° for 6 h. Excess formaldehyde was destroyed with 2N-sodium hydroxide (2 ml), and the mixture was distilled azeotropically. To the distillate was added an excess of saturated ethanolic picric acid, and the precipitate was collected by centrifugation. The picrate (1.05 g; 78%) had m.p. 274-276° (Found: C, 38.7; H, 3.7; N, 18.8. C_7H_16N_2, 3 C_6H_3N_3O requires C, 38.9; H, 3.8; N, 19.1%). The picrate was dissolved in hot 10N-sodium hydroxide (20 ml), the solution was saturated with sodium chloride and then extracted thoroughly with chloroform (6 x 25 ml). The combined extracts were washed twice with 10N-sodium hydroxide (1 ml) and dried over Na_2SO_4 and activated charcoal (0.5 g). Following filtration, dry HCl gas was bubbled through the solution which became turbid. The solvent was evaporated and the residue dried in a vacuum desiccator over KOH overnight. The piperazine hydrochloride (240 mg; 52%) was sublimed at 180° and 2 mmHg in 58% yield. The sublimate had m.p. 236-237° (effervescence); δ(2N-DCI) ν4.2 [7H, multiplet, 3 CH_2 and H(2)], 3.55 [6H, s, CH_3(1) and CH_3(4)] and 2.00 [3H, d, J 6, CH_3(2)] (lit., 219, 3.60 and 2.06); νmax. 3130, 2990, 2940 (CH), 2630, 2610, 2560, 2510, 2490, 2460, 1470 (CH) and 1435 (Found: C, 41.6; H, 9.0; N, 13.7; Cl, 35.3. C_7H_{16}N_2·2 HCl requires C, 41.8; H, 9.0; N, 13.9; Cl, 35.35%).

2S-(+)-1,2,4-Trimethylpiperazine hydrochloride (27.88 mg) in 2.00 N-HCl (2.00 ml) had:

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REFERENCES

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63 Viscontini, M. and Möhlmann, E.,


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Pterins.† Part 2.† Stereochemistry of Catalytic Reduction of 6-Methyl- and 6,7-Dimethyl-pterin and of 2,4-Diamino-6-methylpteridine

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Pterins.† Part 2.1 Stereochemistry of Catalytic Reduction of 6-Methyl- and 6,7-Dimethyl-pterin and of 2,4-Diamino-6-methylpteridine

By Wilfred L. F. Armarego * and Henning Schou, Medical Chemistry Group, The John Curtin School of Medical Research, The Australian National University, Canberra, A.C.T. 2600, Australia

Catalytic addition of two molecules of hydrogen to 7-deuterio-6-trideuteriomethylpterin yields a 0.8 : 1 mixture of cis- and trans-7-deuterio-6-trideuteriomethyl-5,6,7,8-tetrahydropterin. Similar reduction of 2,4-diamino-7-deuterio-6-(partial)trideuteriomethyl-5,6,7,8-tetrahydropteridine gives a 1 : 1 mixture of cis- and trans-2,4-diamino-7-deuterio-6-(partial)trideuteriomethyl-5,6,7,8-tetrahydropteridine. Catalytic reduction of 6,7-dimethyl- and 6,7-bis(trideuteriomethyl)pterin, on the other hand, is stereospecific and forms only the cis-5,6,7,8-tetrahydro-derivatives. Reduction of 6,7-dimethylpterin with sodium in ethanol provides a 1 : 1 mixture of cis- and trans-6,7-dimethyl-5,6,7,8-tetrahydropteridine. The stereochromy of these products was deduced from 1H n.m.r. spectroscopy.

The 5,6,7,8-tetrahydro-derivatives of 6-methyl- (1) and 6,7-dimethyl-pterin (2) are substrates for the monooxygenase enzyme systems which hydroxylate (5)-phenylalanine to (5)-tyrosine,2,3 (5)-tyrosine to (5)-3,4-dihydroxyphenylalanine (dopa),2,4 and (5)-tryptophan to (5)-5-hydroxytryptophan.5 The first-mentioned system, which has been studied in great detail,2,3 consists of at least two separate enzymes: a hydroxylase which oxidises phenylalanine, and an NADPH-requiring dihydropterin reductase which reduces the dihydropterin formed back to its tetrahydro-derivative; and the cycle is then repeated. The tetrahydro-derivatives of the substrates (1) and (2) can replace the natural coenzyme 5,6,7,8-tetrahydrobiopterin (3) very effectively.2,3 Much evidence has been presented in support of the intermediate 'quinonoid' 6,7-dihydropterin (4), which could not be isolated because it rearranges rapidly in the absence of enzymes to the isomeric biologically inactive 7,8-dihydropterin.2,3,5,6 The available data indicate that the chiral centre at C-6 and the chiral or prochiral centre at C-7 are unaffected in the enzymic cycle. We require, however, more direct evidence regarding the relative configuration of the two centres during the cycle. We also want to know the relative and absolute stereospecificities at these two centres when the substrate (1) is reduced by folate reductases.

In order to solve these problems we needed a method to identify the stereochemistry of addition of hydrogen to C-6 and -7 in 6-methyl- and 6,7-dimethyl-pterin. The 1H n.m.r. approach chosen was based on the knowledge that the vicinal coupling constants for the cis-2- and 3-protons (ca. 2.7 Hz) in substituted 1,2,3,4-tetrahydroquinaxalines were consistently smaller than those for the corresponding protons (ca. 8 Hz) in the trans-isomers.7 We describe here the preparation and spectra of 6,7-dimethyl- (5), 6,7-bis(trideuteriomethyl)- (6), 6-methyl- (6), 7-deuterio-6-methyl- (7), 6-trideuteriomethyl- (9), and 7-deuterio-6-trideuteriomethyl- (10) 5,6,7,8-tetrahydropteridines, and of 2,4-diamino-7-deuterio-6-trideuteriomethyl-5,6,7,8-tetrahydropteridine. The relative stereochemistry of hydrogen addition to C-6 and C-7 is not affected in the enzymic cycle. We require,
-7 to form these tetrahydro-derivatives can now be deduced from $^1$H n.m.r. data.

\[
\begin{align*}
1) & \quad R^1 = R^2 = H \\
2) & \quad R^1 = H, R^2 = Me
\end{align*}
\]

\[
\begin{align*}
3) & \quad H N \quad C - CH_3 \\
4) & \quad H N \quad Me
\end{align*}
\]

\[
\begin{align*}
5) & \quad R = Me \\
6) & \quad R = H \\
7) & \quad R = D
\end{align*}
\]

\[
\begin{align*}
8) & \quad R = CD_3 \\
9) & \quad R = H \\
10) & \quad R = D
\end{align*}
\]

The methyl protons of 6,7-dimethylpterin, which is readily prepared from 2,4,5-triamino-6-hydroxypyrimidinium sulphate and biacetyl, are barely exchanged by deuterium in deuterated acid. In deuteriated aqueous base, on the other hand, the exchange is much faster and 6,7-bis(trideuteriomethyl)pterin with >98% isotopic purity is obtained. Catalytic reduction of this product in 3N-hydrochloric acid gave 6,7-bis(trideuteriomethyl)-5,6,7,8-tetrahydropterin hydrochloride (8) in high yield without observable loss of deuterium. The $^1$H n.m.r. spectrum of this compound [see below and Figure 1(c)] shows that the configuration is entirely cis. While this work was in progress Weber and Viscontini demonstrated by a different method that the catalytic reduction of 6,7-dimethylpterin in trifluoroacetic acid gave exclusively the cis-tetrahydro-derivative (5) (see below).

The catalytic reduction of 6-methylpterin to 6-methyl-5,6,7,8-tetrahydropterin provides only one product, although the relative stereochemistry of the hydrogen atoms that add to C-6 and C-7 can be cis or trans. The stereochemistry of the addition can, however, be determined by the reduction of 7-deuterio-6-methylpterin. We have analysed the $^1$H n.m.r. spectrum of 6-methyl-5,6,7,8-tetrahydropterin hydrochloride in D$_2$O. The axial and equatorial C-7 proton signals are clearly separated and their coupling constants with H-6 are observable [see Figure 2(a) and below]. The spectrum of the tetrahydro-derivative of 7-deuterio-6-trideuteriomethylpterin should therefore readily show the steric relation of the protons that have added to C-6 and -7.

Our first approach to the synthesis of 7-deuterio-6-methylpterin was by direct deuteriation of 6-methylpterin. Here, in 6,7-dimethylpterin, we found that deuteriated acid caused very little exchange, other than of the labile hydrogen atoms on the nitrogen atoms. Deuteriated alkali exchanged all the protons on the 6-methyl group, but there was negligible exchange of H-7 under the variety of conditions tried. We then turned to Taylor's unambiguous pteridine synthesis; viz. (11

\[
\begin{align*}
\text{(11; } R_1 = R_2 = H) \quad \text{(12; } R_1 = R_2 = H, R = CO_2Et) \\
\text{(13; } R_1 = R_2 = H) \quad (13) \quad (13; \text{ other combinations})
\end{align*}
\]

Perdeuteriopyruvaldehyde oxime (deuteriated oximinoacetone) (11; $R_1 = R_2 = D$) of high deuterium content was prepared as described from ethyl acetoacetate but using

\[
\begin{align*}
\text{10 E. C. Taylor, K. L. Perlman, I. P. Sword, M. Séquin-Frey,} \\
\text{and P. A. Jacobi, } J. \text{ Amer. Chem. Soc., } 1973, 95, 6407.
\end{align*}
\]

\[
\begin{align*}
\end{align*}
\]

\[
\begin{align*}
\text{12 L. Vanino, 'Handbuch der Praparative Chemie,' Ferdinand Enke Verlag, Stuttgart, 1936, vol. 11, p. 834.}
\end{align*}
\]
completely deuteriated reagents in deuterium oxide. The direct deuterium exchange reactions

![Figure 2](https://example.com/figure2.png)

of the oxime were less satisfactory, and in deuterium oxide containing sodium deuterioxide, the protons of the methyl group of the oxime were exchanged more rapidly than the aldehydic proton. Although almost complete exchange of the aldehydic proton was possible, it was found that, during exchange of the CD₃ group to give back a CH₃ group with aqueous alkali, much self-condensation of the oxime had taken place. This was revealed by an increase in the number of C-methyl signals in the ¹H n.m.r. spectrum. The reaction of ethyl α-aminocynoacetate toluene-p-sulphonate salt with the deuteriated oxime proceeded smoothly and gave 2-amino-6-deuterio-3-ethoxy carbonyl-5-trideuteriomethyl pyrazine 1-oxide (12; R = CO₂Et, R¹ = R² = D) in which only a small percentage of the deuterium on the methyl group and none of the 6-D had been exchanged. The deuteriated ester reacted with guanidine in the presence of sodium methoxide but provided 6-methylpterin 8-oxide (13; R¹ = R² = H) in which almost complete exchange of deuterium at C-7 and in the methyl group had occurred. A repetition of this reaction with deuteriated reagents and deuteriated solvents would be prohibitive in cost. Alternative syntheses were therefore sought. Attempts to prepare the pterin 8-oxide (13; R¹ = R² = H) from 2-amino-3-ethoxy carbonyl-5-methyl pyrazine 1-oxide (12; R = CO₂Et, R¹ = R² = H) by condensation with cyamide or methyl isothiouromium sulphate, or from 2-amino-3-carbamoyl-6-methylpyrazine 1-oxide with cyanamide or cyogen bromide, or from 3-carbamoyl- 3-ethoxy carbonyl-2-amino-5-methylpyrazine with guanidine and sodium methoxide were uniformly unsuccessful. These data imply that in the formation of the pteridine 8-oxide, guanidine reacts first with the nitrile or ester function in (12; R = CN or CO₂Et), and intramolecular cyclization then takes place onto the 2-amino-group. If this is not the case then the pyrazine 1-oxide (12; R = CO₂Et, R¹ = R² = H) should be less reactive than 2-amino-3-ethoxy carbonyl-5-methyl pyrazine because of the stronger basicity of the amino-group in the latter. Predicting that the nitrile function would be more reactive than the ester function in this system, we condensed 2-amino-3-cyano-5-methyl pyrazine with guanidine as above and obtained 2,4-diamino-6-methylpteridine (14; R¹ = R² = H) in good yields (cf. ref. 11). When the reaction was repeated with 2-amino-3-cyano-6-deuterio-5-methyl pyrazine [obtained from the oxime (11; R¹ = R² = D) by deoxygenation of the derived 1-oxide (12; R = CN, R¹ = R² = D)], it provided 2,4-diamino-6-methylpteridine (14) in which C-7 was completely deuteriated and the 6-methyl group was ca. 65% deuteriated. This result is not surprising because we know that the hydrogen atoms on the methyl group in 6-methylpterin are exchanged in the presence of sodium deuterioxide. Catalytic reduction of the diaminopteridine gave 2,4-diamino-7-deuterio-6-(partial)-trideuteriomethyl-5,6,7,8-tetrahydropteridine hydrochloride which had a ¹H n.m.r. spectrum consistent with a product from a mixture of cis- and trans-addition of hydrogen at C-6 and -7 (see below). The integrals indicated that the 6-methyl group was 65% deuteriated.

2,4-Diamino-6-methylpteridine was relatively stable in warm hydrochloric acid but, as in the hydrolysis of 2,4-diamino-6-bromomethylpteridine hydrobromide to 6-bromomethylpterin hydrobromide, ¹H n.m.r. spectrum consistent with a product from a mixture of cis- and trans-addition of hydrogen at C-6 and -7 (see below). The integrals indicated that the 6-methyl group was 65% deuteriated.

2,4-Diamino-6-methylpteridine was relatively stable in warm hydrochloric acid but, as in the hydrolysis of 2,4-diamino-6-bromomethylpteridine hydrobromide to 6-bromomethylpterin hydrobromide, it was converted

---

into 6-methylpterin hydrobromide on heating with aqueous 48% hydrobromic acid. A much more satisfactory preparation of 6-methylpterin involved heating and recrystallising the 2,4-diamino-derivative from 2N-sodium hydroxide, which yielded the sodium salt of 6-methylpterin from which the free base can be isolated in a pure state by acidification. A similar recrystallisation of 2,4-diamino-7-deutero-6-(partial)trideuteriopteridine from aqueous 2N-sodium hydroxide gave 7-deutério-6-methylpterin, whereas heating and recrystallising from 2N-sodium hydroxide gave the deutéro-compound (1; R1 = R2 = D) in excellent yields. Catalytic reduction of these compounds furnished the tetrahydro-derivatives (7) and (10), respectively, without loss of deuterium at C-7.

\[ \text{H N.m.r. data of 5,6,7,8-tetrahydropteridines at 100 MHz} \]

<table>
<thead>
<tr>
<th>5,6,7,8-Tetrahydropterin hydrochloride</th>
<th>CH3</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>cis-6,7-Dimethyl</td>
<td>H-6</td>
<td>4.34</td>
</tr>
<tr>
<td>cis-6,7-Bis(trideuteriomethyl)</td>
<td>H-7</td>
<td>4.43</td>
</tr>
<tr>
<td>trans-6,7-Dimethyl</td>
<td>(J 3.1, 6.7)</td>
<td>4.45</td>
</tr>
<tr>
<td>6-Trideuteriomethyl*</td>
<td>(J 8.3, 6.8)</td>
<td>4.10</td>
</tr>
<tr>
<td>(15; R1 = CHD2, R2 = H2O)</td>
<td>(JAB 3.6, JAD 9.0)</td>
<td>H4</td>
</tr>
<tr>
<td>6-Methyl</td>
<td>(as above and J 6.4)</td>
<td>H4</td>
</tr>
<tr>
<td>6-Methyl #</td>
<td>(JAB 3, JAO 10.0)</td>
<td>H4</td>
</tr>
<tr>
<td>trans-7-Deutero-6-trideuteriomethyl*</td>
<td>H4</td>
<td>4.19</td>
</tr>
<tr>
<td>(16)</td>
<td>(J 9.0)</td>
<td>H4</td>
</tr>
<tr>
<td>cis-7-Deutero-6-trideuteriomethyl*</td>
<td>H4</td>
<td>4.20</td>
</tr>
<tr>
<td>(17)</td>
<td>(as above and J 6.4)</td>
<td>H4</td>
</tr>
<tr>
<td>2,4-Diamino-5,6,7,8-tetrahydropteridine hydrochloride</td>
<td>7-ax</td>
<td>3.84</td>
</tr>
<tr>
<td>6-Methyl</td>
<td>(J 10.2)</td>
<td>4.20</td>
</tr>
<tr>
<td>trans-7-Deutero-6-</td>
<td>(J 10.2)</td>
<td>4.20</td>
</tr>
<tr>
<td>(partial)trideuteriomethyl</td>
<td>(J 14.5)</td>
<td>4.20</td>
</tr>
<tr>
<td>cis-7-Deutero-6-(partial)trideuteriomethyl</td>
<td>4.20</td>
<td>4.20</td>
</tr>
</tbody>
</table>

* Concentration 20 mg in 0.5 ml tetramethylsilane as external standard. # Assignments may be reversed. # Computer-simulated spectrum from the experimental signal positions (by M. J. Whittaker), error ±0.1 Hz. * Data at 270 MHz are almost identical with these although the spectrum appears simpler (see Figure 2). * From ref. 14.

1H N.m.r. Spectra and Stereochemistry.—The spectrum of 6,7-dimethyl-5,6,7,8-tetrahydropterin hydrochloride in deuterium oxide (Figure 1(a) and Table) has eleven lines (theory: fourteen lines) for H-6 and -7, and two doublets for Me-6 and -7. The coupling constants for the methyl doublets (6.7 Hz) are almost identical, making the vicinal coupling constant for H-6 and -7 (3.1 Hz) readily observable. The small coupling constant suggests a cis-stereochemistry consistent with a half-chair conformation (15; R1 = R2 = Me) or equilibrating half-chair conformations (15) = (15a), as has previously been postulated for such systems.7,8,14 The spread of the signals for cis H-6 and -7 (ca. 0.5 p.p.m.) could conceivably be masking signals from the trans-isomer which may be a contaminant. However, the absence of the latter isomer in the crude and purified samples was confirmed by the spectrum of 6,7-bis(trideuteriomethyl)-5,6,7,8-tetrahydropterin hydrochloride [Figure 1(c)] which was prepared under identical conditions. The J value and chemical shifts of the two doublets are consistent with the values derived from the multiplet of the non-deuteriated isomer. Our deductions and those reported7 were made only from the knowledge that the coupling constant observed between H-6 and -7 was small. We confirmed this beyond doubt by preparing a 1:1 mixture of cis- and trans-6,7-dimethyl-5,6,7,8-tetrahydropterin hydrochloride by reduction of 6,7-dimethylpterin with a large excess of sodium in ethanol. The 1H N.m.r. spectra of the isomers were clearly separated [see Figure 1(b)] and the coupling constant between H-6 and -7 in the trans-isomer was significantly larger than that in the cis-isomer (see Table).

The spectrum of 6-methyl-5,6,7,8-tetrahydropterin hydrochloride and its 6-deuterio-derivative in 0.5N-deuterium chloride at 100 MHz had been reported previously13 and the J values were deduced by computation. The quartet for H-7 was assigned from the spectrum of the 6-deuteriated derivative. We attempted unsuccessfully to convert the ABC pattern of signals from H-6, Hax-7 and Heq-7 into a first-order spectrum by varying the solvents [e.g. CF3CO2H, D2SO4, (CD3)2SO, (CD3)2N-CDO, and DCl]; however our spectrum of the hydrochloride in deuterium oxide was similar to the one in 0.5N-deuterium chloride reported. The complicating factor in this spectrum is the further coupling of H-6 with the 6-methyl group which makes the theoretical spectrum of H-6 consist of sixteen lines. In a further attempt to obtain a first-order spectrum we measured this compound...
at 270 MHz [Figure 2(a)]. The signal pattern for H-6 and -7 [i.e. in (15; R¹ = Me, R² = H)] at this field was slightly different from the one at 100 MHz, but there appeared to be very little dispersion of H-6 and Heq-7. The pattern was, however, simplified in the spectrum of

It is now possible, with the knowledge of the above assignments, to determine the stereochemistry of addition of hydrogen to C-6 and -7 in 7-deuterio-6-trideuterio-methylpterin. The spectrum of the tetrahydro-derivative obtained from catalytic reduction [Figure 2(c)] consists of two doublets (trans $f$ 9.0 Hz) and a singlet inside the downfield doublet. The chemical shifts of these signals, when compared with those of 6-trideuterio-methyl-5,6,7,8-tetrahydropterin hydrochloride, confirm that the compound is a mixture consisting of the trans-isomer (a quartet) in the predominant conformation (16) and the cis-isomer (a singlet) in the predominant conformation (17), with almost similar chemical shifts for H₄ and H₅ in the cis-isomer, although the trans- (16) and cis- (17) conformers predominate. The spectra give time-averaged lines for each isomer due to the conformers in equilibrium. The slightly broadened singlet from the cis-isomer in the spectrum [Figure 2(c)] is not centred between the doublet from the trans-isomer and can be explained if the position of equilibrium of the conformers is slightly different in the two isomers. This difference can be caused by the effect of the deuterium atom on the conformational equilibria, or a deuterium isotope effect on the chemical shift of the geminal proton, and is probably not of steric origin. The cis:trans ratio calculated from the integrals is 0.8:1.

The spectrum of 2,4-diamino-6-methyl-5,6,7,8-tetrahydropteridine hydrochloride is quite similar to that of the pterin (6), i.e. an AA'B pattern, and the chemical shift of the C-7 protons can be assigned by inspection from the above knowledge. 2,4-Diamino-7-deutério-6-(partial)-trideuteriometyllpteridine gave on catalytic reduction in 3N-hydrochloric acid the corresponding tetrahydro-derivative, which had a spectrum similar to that of the pterin (10). The cis:trans ratio in this case was ca. 1:1.

The difference in the stereospecificity between the catalytic additions of hydrogen to 6,7-dimethylpterin and to 6-methylpterin deserves some comment. Undoubtedly two reduction steps are involved: addition of one molecule of hydrogen across the 7,8-double bond followed by addition across the 5,6-double bond. We have checked this point with 6-methylpterin by measuring the $^1$H n.m.r. and u.v. spectra of samples withdrawn after the absorption of 0.4, 0.9, 1.5, and 2.0 mol. equiv. of hydrogen. The spectra showed that the ratios of 6-methylpterin to 6-methyl-7,8-dihydropterin to 6-methyl-5,6,7,8-tetrahydropterin were 0.7:1.0:0.0; 0.1:1.0:0.0; 0.0:1.0:0.8; and 0.0:0.0:1.0, respectively. A sample withdrawn after absorption of 1 mol. equiv. of hydrogen was evaporated and the product converted into the dithionite salt. This proved identical with authentic 6-methyl-7,8-dihydropterin dithionite. In 6,7-dimethylpterin addition of hydrogen across the 7,8-double bond is slower than in the above and gave ratios of pterin to dihydropterin to tetrahydropterin of 0.4:1.0:0.4; 0.1:0.8:1.0; and 0.0:0.0:1.0 after absorption of 1.0, 1.5, and 2.0 mol. equiv. of hydrogen, respectively. The intermediate was shown to be 6,7-dimethyl-7,8-dihydropterin
by aeration of a sample at the end of the reduction, giving a u.v. spectrum similar to the one obtained from the sample withdrawn after absorption of 1.5 mol. equiv. of hydrogen. The $^1$H n.m.r. spectra are also consistent with these findings. Aeration of 6,7-dimethyl-5,6,7,8-tetrahydropyridazine is known to furnish the 7,8-dihydropyridazine.$^{18}$ The stereospecificity of cis-addition in 6,7-dimethylpyridazine can be explained by adsorption of the molecule on the reduced catalyst and the addition of one molecule of hydrogen across the 7,8-double bond. Then either the dihydro-substrate is held on the catalyst and a second molecule of hydrogen is added stereospecifically, or more likely it is desorbed, and readsorbed stereospecifically because of the encumbrance of the 7-methyl group (i.e. structure (19) would be more favoured in the transition state than structure (18)). In the case of 6-methylpterin, after the 7,8-dihydro-compound is formed it must be released into the solution and readsorbed on the catalyst, almost randomly because of lack of steric hindrance near C-7, and reduced further across N(5)–C(6).

**Experimental**

Elemental analyses were determined by the Australian National University Analytical Service Unit; values for H + D were calculated as before.$^{17}$ i.r. spectra (KBr) were measured with a Unicam SP 1000 spectrometer, and u.v. spectra with a Unicam SP 1800.$^1$ H N.m.r. spectra were obtained with Varian T60A and HA100 spectrometers (tetramethylsilane as internal or external lock). 270 MHz Spectra were measured with a Bruker HFX-270 spectrometer by the National NMR Centre (Dr. A. J. Jones). J Values are in Hz. Mass spectra (by Dr. J. K. MacLeod and staff) were measured with an A.E.I. MS9 instrument. Values are in Hz. Mass spectra (by Dr. J. K. MacLeod and staff) were measured with an A.E.I. MS9 instrument.}

6,7-Dimethylpterin (7 g) was converted into the sodium salt (82%) by recrystallisation from 2N-sodium hydroxide (150 ml). The salt was washed with a little cold water then ethanol and dried at 100 °C, and had m.p. >360 °C (decomp.) (Found: C, 34.5; H, 3.9; N, 32.6; Na, 11.1. C$_7$H$_5$N$_6$NaO$_2$ requires C, 34.1; H, 3.8; N, 32.8; Na, 10.8%). The sodium salt of 6,7-bis(trideuteriomethyl)pterin was prepared in 70% yield by heating 6,7-dimethylpterin (250 mg) at 100 °C in 2N-sodium deuterioxide in deuterium oxide (25 ml) for 24 h in a sealed tube and isolated as above. It had no proton n.m.r. signals in 2N-sodium deuterioxide or 2N-deuterium chloride.

6-Methylpterin Sodium Salt.—Crude 6-methylpterin, prepared from 2,5,6-triaminopyridine-4-sulphonic acid and sodium hydrosulphite as before$^{18}$ but on a 130 g scale, was shown by $^1$H n.m.r. spectroscopy in 2N-DCI–D$_2$O to contain 36% of 7-methylpterin (82.77 (7-Me), 2.80 (6-Me), 8.81 (6-H), and 8.93 (7-H)). The pure sodium salt of 6-methylpterin was obtained by recrystallisation of the mixture from 10 parts of 2N-sodium hydroxide (60% recovery) and had m.p. >360 °C (decomp.) (Found [after drying at 150 °C for 12 h]: C, 41.4; H, 3.3; N, 34.4; Na, 11.3). C$_7$H$_5$N$_6$NaO$_2$·0.25H$_2$O requires C, 41.3; H, 3.2; N, 34.6; Na, 11.3%). The free base was prepared by acetylation of an aqueous solution of the salt, and collecting and washing (H$_2$O and EtOH) by centrifugation because conventional filtration was exceedingly slow. This compound and its tetrahydro-derivative were identical with samples from an unequivocal synthesis.$^{19}$

6-Trideuteriomethylpterin Sodium Salt.—6-Methylpterin (1 g) in 2N-sodium deuterioxide (70 ml) was heated in a bomb at 100 °C for 24 h. The sodium salt (600 mg) that crystallised on cooling was collected, washed with a little water and ethanol, and dried. It had m.p. >360 °C (decomp.) (Found: C, 38.7; H + D, 4.7; N, 32.9; Na, 10.7. C$_7$H$_5$D$_2$N$_6$NaO$_2$·0.5H$_2$O requires C, 38.8; H + D, 4.7; N, 33.2; Na, 10.9%). Prolonged heating of the sodium deuterioxide solution at 120 °C did not cause H-7 to be displaced by deuterium.

7-Deuterio-6-methylpterin Sodium Salt.—2,4-Diamino-7-deuterio-6-(partial)trideuteriomethylpteridine (200 mg; see below) in 2N-sodium hydroxide (75 ml) was stirred at 100 °C for 9 h. The solution was concentrated until a solid crystallised, and was cooled. The yellow sodium salt (120 mg) was collected as above. A further 60 mg of salt was obtained from the mother liquors. The u.v. spectra and t.l.c. properties were identical with those of authentic non-deuteriated 6-methylpterin, and only a sharp 6-methyl signal was present in the $^1$H n.m.r. spectrum (Found: Na, 11.3). C$_7$H$_5$D$_4$N$_6$NaO$_2$ requires Na, 11.5%).

Deuteriated Hydroxyiminoacetone.—Ethyl acetoacetate (28.3 g) was added to a solution of sodium (4.6 g, 1 mol. equiv.) in deuterium oxide (300 ml) followed by sodium nitrite (12.2 g). The mixture was set aside for 24 h, acidified to pH 1 with concentrated hydrochloric acid, and extracted thoroughly with ether and dried (Na$_2$SO$_4$). Evaporation gave the deuteriated oxime (14.5 g, 87%), which was sublimed at 50 °C and 0.5 mmHg, and had m.p. 65–66 °C (lit, 19 65 °C 0 and EtOH) by centrifugation because

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pyrazine oxide which was removed by dissolving the lysed further by prolonged heating in sulphuric acid. The product was poured into water and neutralised with sublimed at 180-190 °C and 0.1 mmHg to give the oxide. Amino-3-cyano-5-methylpyrazine 1-Oxide—The preceding deuteriated hydroyminoacetic acid (5.8 g) and α-aminomalononitrile toluene-p-sulphonate salt in (16.9 g) in propanol-2-ol (100 ml) were stirred at 25 °C for 4 h. The pyrazine oxide that separated contained some toluene p-sulphonic acid which was removed by dissolving the mixture in water, adjusting the pH to 9, and extracting with chloroform. Evaporation gave the deuteriated pyrazine hydrochloride, m.p. >250 °C (decomp.), its u.v. spectrum was identical with that of the non-deuteriated pyrazine (Found: C, 56.6; H, 4.8; N, 33.5% [1 H n.m.r. data in Figure 1(b) and the Table].) was prepared by catalytic reduction as above. The u.v. spectra and t.l.c. behaviour of these salts were identical with those of authentic 6-methyl-5,6,7,8-tetrahydropteridine hydrochloride. 6-Trideuteriomethyl-5,6,7,8-tetrahydropteridinhydrochloride.—This derivative, m.p. >260 °C (decomp.) (Found: C, 31.2; H, 7.0; Cl, 24.8. C_{7}H_{12}D_{8}N_{5}O_{1}·HCl·H_{2}O requires C, 31.0; H, 6.65; Cl, 24.7% was prepared by catalytic reduction of 6-trideuteriomethylpteridine as above; the 1 H n.m.r. spectrum is in Figure 2(b) and in the Table. 2-Amino-3-cyano-6-deuterio-5-(partial) trideuteriomethylpyrazine 1-Oxide.—In 2-Amino-3-cyano-5-methylpyrazine 1-oxide (5 g) in sulphuric acid (1.8: 25 ml) was heated at 100 °C for 15 min; the product was poured into water and neutralised with aqueous ammonia, and the yellow solid was collected and sublimed at 180-190 °C and 0.1 mmHg to give the amide (84%), m.p. 230-230.5 °C (lit. 219-219 °C, lit. 235-236 °C), as prepared from α-aminoacetoaceticamide and hydroyminoacetic acid; \( \max \ \nu \ (\text{cm}^{-1}; \text{lit.} 1160, 1695, 1670 (\text{amide}), 1620 (\text{C}=\text{N}), 1170 (\text{N}=\text{O}) \) (Found: C, 46.7; H, 3.6; N, 33.6%.) The amide could not be hydrolysed further by prolonged heating in sulphuric acid. 2-Amino-3-carbamoyl-5-methylpyrazine 1-Oxide.—2-Amino-3-cyano-5-methylpyrazine 1-oxide (5 g) in sulphuric acid (1.8: 25 ml) was heated at 100 °C for 15 min; the product was poured into water and neutralised with aqueous ammonia, and the yellow solid was collected and sublimed at 180-190 °C and 0.1 mmHg to give the amide (84%), m.p. 230-230.5 °C (lit. 219-219 °C, lit. 235-236 °C), as prepared from α-aminoacetoaceticamide and hydroyminoacetic acid; \( \max \ \nu \ (\text{cm}^{-1}; \text{lit.} 1160, 1695, 1670 (\text{amide}), 1620 (\text{C}=\text{N}), 1170 (\text{N}=\text{O}) \) (Found: C, 46.7; H, 3.6; N, 33.6%.) The amide could not be hydrolysed further by prolonged heating in sulphuric acid. 2-Amino-3-cyano-6-deuterio-5-(partial) trideuteriomethylpyrazine 1-Oxide.—The preceding deuteriated pyrazine (5 g) was added to a pre-reduced suspension of platinum oxide in 3N-hydrochloric acid (250 ml) and shaken with hydrogen at 20 °C and 720 mmHg. After absorption of the theoretical amount of hydrogen (3 h), the catalyst was filtered off, and the filtrate evaporated. The residue was recrystallised from ethanol containing a little ethanolic hydrogen chloride and gave (quantitative) the tetrahydropteridine hydrochloride, m.p. >230 °C (decomp.), \( \nu \max \ (\text{cm}^{-1}; \text{lit.} 219 (\log e 4.21) \) and 274 nm (4.15) (Found (after drying at 100 °C for 6 h): C, 21.3; H, 5.0; Cl, 45.2. C_{12}H_{13}D_{8}N_{10} requires C, 21.3; H, 5.2; Cl, 45.0%). The HCl content decreased on further heating but the salt darkened in colour. The 1 H n.m.r. data are in the Table. cis-and trans-2,4-Diamino-7-deuterio-6-(partial) trideuteriomethyl-5,6,7,8-tetrahydropteridinhydrochloride.—This mixture was prepared as above from the preceding deuteriated diaminopteridine and had m.p. 230-240 °C (decomp.); its u.v. spectrum was identical with that of the non-deuteriated pteridine salt (Found: C, 27.7; H, 6.8; Cl, 27.1. Calc. for C_{7}H_{12}D_{5}N_{6}·2.3HCl·2H_{2}O: C, 27.8; H, 6.8; Cl, 27.0%). 6-Trideuteriomethyl-5,6,7,8-tetrahydropteridinhydrochloride.—This derivative, m.p. >260 °C (decomp.) (Found: C, 31.2; H, 7.0; Cl, 24.8. C_{7}H_{12}D_{8}N_{5}O_{1}·0.9HCl·H_{2}O requires C, 31.0; H, 6.65; Cl, 24.7% was prepared by catalytic reduction of 6-trideuteriomethylpteridine as above; the 1 H n.m.r. spectrum is in Figure 2(b) and in the Table. Similarly cis- and trans-7-deuterio-6-methyl-5,6,7,8-tetrahydropteridine hydrochloride, m.p. >290 °C (decomp.) (Found: C, 30.5; H, 6.1; Cl, 22.9. Calc. for C_{12}H_{14}D_{8}N_{5}O_{1}·1.6HCl·1.5H_{2}O: C, 30.7; H, 6.2; Cl, 23.0% and cis- and trans-7-deuterio-6-trideuteriomethyl-5,6,7,8-tetrahydropteridinhydrochloride, m.p. >260 °C (decomp.) (Found: C, 34.1; H, 6.6; Cl, 23.6. Calc. for C_{7}H_{12}D_{5}N_{6}O_{1}·0.6HCl: C, 34.4; H, 6.8; Cl, 23.7%) 1 H n.m.r. spectrum in Figure 2(c) and the Table] were prepared by catalytic reduction as above. The u.v. spectra and t.l.c. behaviour of these salts were identical with those of authentic 6-methyl-5,6,7,8-tetrahydropteridinhydrochloride. The HCl and H_{2}O contents of the crystals varied with the drying conditions and the nitrogen figures were all consistently ca. 1% too low. cis-6,7-Bis(trideuteriomethyl)-5,6,7,8-tetrahydropteridinhydrochloride.—This derivative, m.p. >300 °C (decomp.) (Found: C, 35.0; H, 7.8; Cl, 24.6; N, 25.1. C_{12}H_{14}D_{8}N_{5}O_{1}·1.6HCl·0.25H_{2}O requires C, 34.9; H, 7.3; Cl, 24.6; N, 25.4%) was prepared by catalytic reduction of 6,7-bis(trideuteriomethyl)pterin sodium salt as above and had the same u.v. and t.l.c. properties as the authentic non-deuterated salt. The 1 H n.m.r. spectrum is in Figure 1(c) and the Table. cis- and trans-6,7-Dimethyl-5,6,7,8-tetrahydropteridinhydrochloride.—To the sodium salt of 6,7-dimethylpterin (426 mg) under dry nitrogen in boiling ethanol was added sodium until the u.v. spectrum of a sample at pH 2 indicated that reduction was complete. A total of 17 g of sodium was added during a reflux period of 48 h. The solution was cooled in an ice-bath and acidified with ethanolic hydrochloride.
(120 ml) under nitrogen. Sodium chloride was removed by repeated concentration and filtration. The ca. 1 : 1 mixture of cis- and trans-6,7-dimethyltetrahydropterin hydrochloride (282 mg) obtained from the mother liquors was crystallised from ethanol containing a little ethanolic hydrogen chloride under nitrogen. The u.v. data of the mixture, m.p. > 250 °C (decomp.), were identical with those of the cis-isomer; the $^1$H n.m.r. spectrum is in Figure I(b) and the Table (Found: C, 36.2; H, 5.8; Cl, 24.8. Calc. for $C_8H_{15}N_5O_1.85HCl.0.1H_2O$: C, 36.3; H, 5.7; Cl, 24.8%). The trans-isomer was clearly less stable to aerial oxidation than the cis-isomer, with a $t_1$ value at 20 °C and pH 2 (analyt. 219 nm) of 40 min, to be compared with 5.2 h for the pure cis-isomer. Chromatographic separation of the mixture has not yet been achieved.

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