Specificity of Aldehyde Oxidase Towards
N-Heterocyclic Cations

Oxidation of quinolinium and related cations by aldehyde oxidase in vitro; the isolation of two products formed simultaneously from a single substrate

by

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Abstract

Aldehyde oxidase catalysed oxidation of various quinolinium and related cations has been studied in vitro. Oxidation products were identified by comparison of their spectral and chromatographic characteristics with those of authentic compounds. The N-heterocyclic cations and quinolones used required synthesis.

Incubation of N-methylquinolinium, N-methyl-7,8-benzoquinolinium and N-phenylquinolinium yielded the corresponding 2- and 4-quinolones simultaneously. The ratio of 2- to 4-quinolone formation was found to be species dependent; the proportion of 4-quinolone was greater with guinea pig enzyme than with rabbit enzyme.

Incubation of N-methyl-4-methylquinolinium, N-methyl-4-phenylquinolinium and N-methylphenanthridinium produced the expected 2-quinolones. Cations substituted adjacent to the ring nitrogen, i.e. N-methyl-2-methylquinolinium, N-methyl-2-phenylquinolinium and N-phenyl-2-phenylquinolinium, were oxidised to the corresponding 4-quinolones.

Kinetic constants were determined spectrophotometrically. The $K_m$ values obtained with rabbit enzyme ranged from $1.6 \times 10^{-3}$ M for N-methylquinolinium to $<10^{-5}$ M for N-phenyl-2-phenylquinolinium. Quaternary compounds were found to be better substrates than their non-quaternary counterparts, except for N-methylisouquinolinium and N-methylphenanthridinium. In general, guinea pig aldehyde oxidase was shown to have a greater affinity for N-heterocyclic cations than rabbit enzyme.

The substrate binding site has been discussed in the light of the results outlined below.

Oxidation of N-methyl-4-phenylquinolinium (to the 2-quinolone) was competitively inhibited by N-methyl-2-phenylquinolinium (which yields the 4-quinolone), indicating that both these cations interact at the same active site. The ratio of 2- to 4-quinolone production from N-methylquinolinium was constant under various conditions, including purification of the enzyme but changed at high pH or in the presence of N-methylphenanthridinium.

Inhibition studies indicated that both quaternary and non-quaternary compounds act at the same site on the enzyme. $K_m$ and $V_{max}$ values for phthalazine, N-methyl-2-phenylquinolinium and N-methylquinolinium were determined over the pH range 5.4 to 10.2. In each case, results indicated that the enzyme has an ionisable group at the active site with a $pK$ ca. 8.

Aldehyde oxidase was shown to catalyse the dehydrogenation of the pseudobases 3,4-dihydro-4-hydroxy-3-methyl-2-quinazolinone and 3,4-dihydro-4-hydroxy-3-methylquinazoline.
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<tbody>
<tr>
<td>5.8</td>
<td>Inhibition by the competing substrate, 6-methylpurine, of the rabbit aldehyde oxidase catalysed oxidation of 3,4-dihydro-4-hydroxy-3-methyl-2-quinazolinone</td>
<td>233</td>
</tr>
<tr>
<td>5.9</td>
<td>The u.v. spectrum of 3,4-dihydro-4-hydroxy-3-methyl-quinazoline and of the product of its oxidation catalysed by aldehyde oxidase</td>
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</tr>
<tr>
<td>5.10</td>
<td>The u.v. spectrum of 3-methyl-4-quinazolinone</td>
<td>235</td>
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</tbody>
</table>
Acknowledgements

I am indebted to Mr. Godfrey Stell and Dr. Christine Beedham for directing and supervising this research and for their valued advice and guidance in the preparation of this manuscript. I also wish to record my thanks to Professor D.W. Mathieson for his continued interest and helpful advice given throughout this study.

My sanity during the long hours in the laboratory was maintained by the friendship of my colleague Christine Johnson, to whom I am very grateful. A thank you also to other members of the Pharmacy Department team, in particular Dr. John Greenhill for his advice concerning organic syntheses.

The patience, understanding and support given by my husband Paul, over the period of this research, deserves a very special thank you. This thesis is dedicated to my parents, as an acknowledgement of the unfailing support and encouragement they have given me throughout my education.

Finally, I thank Elaine Goodrich for typing this thesis and the S.E.R.C. for financial support.
CHAPTER 1

Introduction

Oxidation plays a major role in the biotransformation of foreign compounds. Although the microsomal mono-oxygenase system is of prime importance in this respect, enzymes present in the cytosol can also contribute to this process. This thesis is concerned with one such enzyme, aldehyde oxidase, and in particular, its ability to catalyse the oxidation of N-heterocyclic cations.

ALDEHYDE OXIDASE (EC 1.2.3.1)

Aldehyde oxidase is a metalloenzyme containing molybdenum and non-haem iron. It belongs to a small group of closely related enzymes collectively termed "Molybdenum Hydroxylases" which also includes xanthine oxidase and xanthine dehydrogenase. These enzymes have similar molecular properties but differ in substrate specificity.

The name, aldehyde oxidase, originates from the fact that one of the first observed catalytic activities of the enzyme was the oxidation of aldehydes to carboxylic acids. Subsequently it was also found to catalyse the introduction of an oxygen atom into a diverse range of nitrogen containing heterocyclic compounds, e.g. the compounds below are all good substrates.

\[
\begin{align*}
\text{N-Ethylquinolinium chloride} & \quad \text{2-Hydroxy-5-fluoropyrimidine} \\
\end{align*}
\]

\[
\begin{align*}
\text{N-Ethylquinolinium chloride}^3 \quad \text{2-Hydroxy-5-fluoropyrimidine}^4
\end{align*}
\]
Oxidation of these compounds occurs at an electron deficient carbon atom adjacent (α) to the ring nitrogen.

The overall reaction catalysed by aldehyde oxidase and related enzymes may be represented thus:

The oxygen introduced into the reducing substrate (RH) is derived from water; this is in contrast to reactions catalysed by the microsomal mono-oxygenase system where it is supplied by molecular oxygen. The electron acceptor (A) for aldehyde oxidase under physiological conditions is oxygen (thus the designation "oxidase") but in vitro a number of compounds can adequately perform this role. The enzyme functions by undergoing a cycle of reduction by the reducing substrate (RH) and then reoxidation by an electron acceptor (oxidising substrate), with the resultant conversion of RH to ROH.
1.1 **Distribution**

In common with other molybdenum hydroxylases, aldehyde oxidase is widely distributed in nature, being present in species as diverse as sea anemone and man. The highest levels of the enzyme are found in the vertebrates where it tends to be localised in the liver. In general, herbivores have a higher activity of aldehyde oxidase than carnivores, with rabbit liver being a particularly rich source of the enzyme. Aldehyde oxidase from rabbit liver appears to oxidise a wider range of substrates than that derived from other mammals such as rat and man. Recently a form of the enzyme with a relatively narrow substrate specificity has been isolated from guinea pig granulocytes. Electrophoretic studies by Holmes demonstrated the presence of two isoenzyme forms of aldehyde oxidase in mouse. The most abundant isoenzyme was located in the liver and pancreas only, with low levels of a second isoenzyme in the stomach, testes, ovary, heart and lung as well as in the liver and pancreas.

1.2 **Reducing Substrate Specificity**

1.2.1 **Uncharged substrates**

The oxidation of N-heterocycles and aldehydes is catalysed by aldehyde oxidase and in general N-heterocycles are found to be the better substrates. For example, quinoline-6-aldehyde is oxidised in the heterocyclic ring prior to the oxidation of the aldehyde substituent.
It is doubtful whether aldehyde oxidase plays any significant role in the metabolism of aldehydes \textit{in vivo}, as they have a much greater affinity for the unrelated, NADH-dependent aldehyde dehydrogenase.\textsuperscript{14,15} By comparison, aldehyde oxidase is known to be involved in the biotransformation of a number of therapeutically important heterocycles, \textit{e.g.} the antimalarial quinine\textsuperscript{16} and the cytotoxic agent 6-methylthiopurine.\textsuperscript{17} However, despite considerable discussion\textsuperscript{18-20} no clear biological role has yet emerged for this enzyme. It has been suggested that aldehyde oxidase may have evolved to detoxify certain naturally occurring alkaloid type compounds, thus explaining the high levels of this enzyme found in herbivores.\textsuperscript{19}

The ability of aldehyde oxidase to catalyse the oxidation of N-heterocycles was first discovered by Knox in 1946\textsuperscript{21} who, using a relatively crude preparation of the enzyme, found a number of substituted quinolines to be substrates. Investigation of the oxidation products for a number of these compounds revealed that oxidation had occurred at the carbon atom adjacent to the ring nitrogen in each case.

Krenitsky \textit{et al.}\textsuperscript{22} made a comprehensive comparative study of the substrate specificities of xanthine oxidase and aldehyde oxidase. They compared the initial rates of oxidation of more than fifty compounds and found both enzymes to have a preference for compounds with a substituted pyrimidine ring structure and that substrate specificity was greatly influenced by the position and type of substituent as illustrated in Table 1.1. Furthermore, it was shown that while aldehyde oxidase and xanthine oxidase behave in a broadly similar manner towards some compounds, they exhibit very contrasting behaviour with respect to others. 6-Mercaptopurine is oxidised more rapidly than 6-hydroxypurine by aldehyde oxidase, while the reverse is true with xanthine oxidase. Several other important examples of this contrasting behaviour have since been documented by other workers.
Table 1.1. A comparison of the structure-substrate activity relationships of bovine milk xanthine oxidase and rabbit liver aldehyde oxidase.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Substrate</th>
<th>Rate*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Xanthine Oxidase</td>
</tr>
<tr>
<td>(R₁=R₂=H)</td>
<td>Pyrimidine</td>
<td>&lt;3</td>
</tr>
<tr>
<td>(R₁=H R₂=OH)</td>
<td>2-Hydroxypyrimidine</td>
<td>28</td>
</tr>
<tr>
<td>(R₁=OH R₂=H)</td>
<td>4-Hydroxypyrimidine</td>
<td>130</td>
</tr>
<tr>
<td></td>
<td>Imidazole</td>
<td>&lt;3</td>
</tr>
<tr>
<td>(R₁=R₂=H)</td>
<td>Purine</td>
<td>100</td>
</tr>
<tr>
<td>(R₁=SCH₃ R₂=H)</td>
<td>6-Mercaptopurine</td>
<td>17</td>
</tr>
<tr>
<td>(R₁=CN R₂=H)</td>
<td>6-Cyanopurine</td>
<td>8</td>
</tr>
<tr>
<td>(R₁=OH R₂=H)</td>
<td>6-Hydroxypurine</td>
<td>130</td>
</tr>
<tr>
<td>(R₁=OH R₂=OH)</td>
<td>2,6-Dihydroxypurine</td>
<td>170</td>
</tr>
<tr>
<td></td>
<td>Pteridine</td>
<td>&lt;3</td>
</tr>
<tr>
<td>(R₁=R₂=H)</td>
<td>4-Hydroxypteridine</td>
<td>56</td>
</tr>
<tr>
<td>(R₁=OH R₂=H)</td>
<td>4-Hydroxypteridine</td>
<td>86</td>
</tr>
<tr>
<td>(R₁=OH R₂=NH₂)</td>
<td>2-Amino-4-hydroxypteridine</td>
<td>100</td>
</tr>
</tbody>
</table>

* Initial rates at 24°C, pH 6.8, electron acceptor ferricyanide. Substrate concentration for A.O. 2.1 mM, X.O. 0.07 mM. Rates relative to purine rate. Actual rate with purine for A.O. = 250 nmoles/min/mg, for X.O. = 35 nmoles/min/mg.
Pteridine is a good substrate for both enzymes, yet 2,4-diaminopteridine is only oxidised by aldehyde oxidase. The antineoplastic drug methotrexate, which is a 6-substituted 2,4-diaminopteridine is readily oxidised at position 7 by rabbit liver aldehyde oxidase but is an inhibitor of xanthine oxidase.

Both enzymes catalyse the oxidation of allopurinol to alloxanthine. This oxidation product remains bound to xanthine oxidase forming a tight, inhibitory complex, whereas no such effect occurs with aldehyde oxidase.

Allopurinol is used in the treatment of gout and hyperuricemia by virtue of its ability to inhibit xanthine oxidase and thus prevent a build-up of uric acid.
Substrates such as purines and pteridines can offer multiple potential sites for hydroxylation. Aldehyde oxidase and xanthine oxidase can also differ with regard to site of substrate oxidation. For example, xanthine oxidase catalyses the oxidation of purine (structure shown in Table 1.1) initially in the 6-position followed by subsequent conversion to 2,6-dihydroxypurine (xanthine) and finally 2,6,8-trihydroxypurine (uric acid), whereas purine is oxidised exclusively in the 8-position by aldehyde oxidase.

Although the monoazine pyridine is not a substrate for aldehyde oxidase, the presence of a fused benzene ring produces good substrates for the enzyme, e.g. quinoline and isoquinoline. Stubley et al. reported that whilst the monocyclic diazines (pyrazine, pyrimidine and pyridazine) are inefficient substrates, the corresponding benzodiazines below are good substrates for aldehyde oxidase.

![Chemical Structures](image)

Quinoxaline (Benzopyrazine) Quinazoline (Benzopyrimidine) Cinnoline (Benzopyridazine) Phthalazine (Benzopyridazine)

Of these compounds, only phthalazine and quinazoline showed any reasonable reaction in the presence of xanthine oxidase. Xanthine oxidase appears to function more efficiently with diazines fused with an additional five-membered heterocyclic ring as in purine.

Phenanthridine, one of the best substrates reported for aldehyde oxidase \( (K_m < 10^{-6} \text{ M}) \), is not a substrate for xanthine oxidase which
thus further exemplifies the difference in behaviour of these two enzymes.

1.2.2 N-Heterocyclic cations

Aldehyde oxidase catalysed oxidation of aromatic heterocyclic compounds usually occurs at a carbon atom adjacent to a ring nitrogen and involves nucleophilic attack. Quaternisation of the ring nitrogen, which makes the adjacent carbon even more susceptible to nucleophilic attack, appears to facilitate this reaction. Thus N¹-methylnicotinamide is a reasonable substrate whereas the enzyme shows little activity towards nicotinamide or pyridine. Similarly studies with N-methyl- and N-ethyl-quinolinium salts have shown these compounds to be oxidised at a much faster rate than quinoline and to give rise to the corresponding 2-quinolone. Rajagopalan and Handler reported the \( K_m \) values of these quinolinium compounds to be of the order of \( 10^{-4} \) M (at pH 7.8) compared to \( 3 \times 10^{-3} \) M for quinoline. With increase in pH, \( K_m \) values obtained for N-heterocyclic cations decrease, while \( K_m \) values for uncharged substrates increase.

Xanthine oxidase does not readily catalyse the oxidation of N-heterocyclic cations below pH 9 and even under quite alkaline conditions they are still very poor substrates, e.g. \( K_m \) for N¹-methylnicotinamide is \( 1.2 \times 10^{-2} \) M at pH 9.6. Despite this limitation, a number of N-heterocyclic cations have been investigated with xanthine oxidase, including various quinolinium, benzoquinolinium and pyridinium compounds whereas studies with aldehyde oxidase have been confined to only a few cations. In a similar manner to that observed with uncharged substrates, specificity differences are found between these two enzymes towards N-heterocyclic cations, e.g. trigonelline (N-methyl 3-pyridine carboxylic acid) is not oxidised by aldehyde oxidase but it can be oxidised by xanthine oxidase; phenazine methosulphate is a substrate.
for aldehyde oxidase only.\textsuperscript{33,37} Substitution of an ethyl instead of a methyl group at the nitrogen of quinoline has very little effect upon $K_m$ with aldehyde oxidase\textsuperscript{3} but with xanthine oxidase the effect of this substitution is to increase the $K_m$ twenty-fold.\textsuperscript{33}

As previously stated, the position of enzymatic oxidation is generally at the carbon adjacent to nitrogen. Exceptions to this do sometimes occur however, \textit{e.g.} the product of 1-(4-pyridyl)pyridinium oxidation is 1-(4-pyridyl)-4'-pyridone with both enzymes.\textsuperscript{3,33}

Phenazine methosulphate has no carbon adjacent to nitrogen available for oxidation and the product with aldehyde oxidase is 10-methylphenaz-3-one.\textsuperscript{37}
Such exceptions are not restricted to quaternary compounds: cinnoline has been shown to be oxidised to 4-hydroxycinnoline with aldehyde oxidase.\textsuperscript{29}

Aldehyde oxidase\textsuperscript{*} catalysed oxidation of N\textsuperscript{1}-methylnicotinamide has received considerable attention. Originally it was thought to give only one oxidation product, \textit{i.e.} N-methyl-2-pyridone-5-carboxamide,\textsuperscript{38} but more recently it has been demonstrated that the concomitant formation of a second oxidation product, N-methyl-4-pyridone-5-carboxamide, occurs with this enzyme both \textit{in vitro} and \textit{in vivo}.\textsuperscript{39,40} This is the only example reported of one substrate giving rise to two oxidation products \textit{simultaneously} with aldehyde oxidase. The ratio of 2-pyridone to 4-pyridone formation depends on the species from which aldehyde oxidase is derived.\textsuperscript{39} The

<table>
<thead>
<tr>
<th>Animal</th>
<th>Ratio 2:4 pyridones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td>100</td>
</tr>
<tr>
<td>Guinea Pig</td>
<td>31</td>
</tr>
<tr>
<td>Hamster</td>
<td>2.1</td>
</tr>
<tr>
<td>Calf</td>
<td>25</td>
</tr>
<tr>
<td>Hog</td>
<td>39</td>
</tr>
<tr>
<td>Mouse</td>
<td>1.6</td>
</tr>
<tr>
<td>Rhesus Monkey</td>
<td>34</td>
</tr>
</tbody>
</table>

* Sometimes referred to in the literature as N\textsuperscript{1}-methylnicotinamide oxidase.
ratio for a given species remained constant under a variety of conditions, which was interpreted as being indicative of a single enzyme capable of catalysing the oxidation of $N^1$-methylnicotinamide at two alternate positions.\textsuperscript{39}

Genetic variants of liver aldehyde oxidase were identified in several strains of inbred mice, differing in their activity towards $N^1$-methylnicotinamide with respect to: (i) specific activities, (ii) $K_m$ values and (iii) ratio of 2- to 4-pyridones formed.\textsuperscript{41-43} Investigators were able to make use of these differences to do cross-breeding experiments and results showed that the synthesis of 2- and 4-pyridones were controlled by a single autosomal gene functioning in a codominant manner. The $K_m$ values for 2- and 4-pyridone formation were the same (for each variant) and further kinetic analysis provided evidence that each variant differed in the structural gene of a single enzyme.\textsuperscript{41,42}

Felsted et al.\textsuperscript{44} provided further evidence for the "one enzyme" hypothesis by finding that homogeneous preparations of aldehyde oxidase from either rabbit or hog, catalysed the oxidation of $N^1$-methylnicotinamide to the 2- and 4-pyridones in the same ratio that had previously been observed \textit{in vivo}. These workers also showed that replacement of the N-methyl group by a bulkier propyl group resulted in a greater proportion of the 4-pyridone being produced.

Investigations of $N^1$-methylnicotinamide oxidation using rat liver preparations showed this species to be anomalous in that 4-pyridone formation was more heat labile, more sensitive to inhibition by menadione or 8-hydroxyquinoline \textsuperscript{45} and differed with respect to $K_m$ when compared to 2-pyridone formation.\textsuperscript{39} The pyridone ratio in the urine should reflect the functionality of liver aldehyde oxidase; however, in rat and man the urinary pyridone ratio is not always identical to the ratio observed.
Further work led Stanulović and Chaykin to conclude that in mammals a single enzyme, aldehyde oxidase, is capable of catalysing the oxidation of N\textsuperscript{1}-methylnicotinamide to two alternate products and that in man and rat, xanthine oxidase can contribute to the formation of some additional 2-pyridone. However, it must be pointed out that xanthine oxidase is also present in other mammals studied, e.g. mouse, which have not been shown to exhibit any anomalous behaviour with respect to N\textsuperscript{1}-methylnicotinamide oxidation.

The anomalous behaviour of rat is very interesting in view of some recent developments. Using rat liver, Ohkubo and Fujimira eluted three separate fractions with N\textsuperscript{1}-methylnicotinamide activity from a DEAE column. The first fraction corresponded to xanthine oxidase and gave rise to 2-pyridone only. The second fraction was capable of both 2- and 4-pyridone production but the third fraction produced only 4-pyridone. In 1983, fraction two (1-CH\textsubscript{3}Nmd oxidase I) and three (1-CH\textsubscript{3}Nmd oxidase II) were separated and purified. These oxidases were distinguishable by their substrate specificity and their immunological and enzymological characteristics. 1-CH\textsubscript{3}Nmd oxidase I has a broad substrate specificity whereas 1-CH\textsubscript{3}Nmd oxidase II has only been shown to catalyse the oxidation of N\textsuperscript{1}-methylnicotinamide. 1-CH\textsubscript{3}Nmd oxidase II was also found to be much more heat labile and more sensitive to inhibition by menadione and 8-hydroxyquinoline. This apparent lability of 1-CH\textsubscript{3}Nmd oxidase II over oxidase I might explain the previous in vitro observations that with man and rat the ability to form 4-pyridone was decreased as incubation time increased.

The $K_m$ values given for 2- and 4-pyridone formation from 1-CH\textsubscript{3}Nmd oxidase I were very similar, i.e. 5.8 and 5.4 x 10\textsuperscript{-4} M respectively, while that for 4-pyridone formation from 1-CH\textsubscript{3}Nmd oxidase II was stated to be 1.0 x 10\textsuperscript{-4} M. These results suggest that the anomalous behaviour of the rat may be due to additional oxidation of N\textsuperscript{1}-methylnicotinamide by 1-CH\textsubscript{3}Nmd oxidase II,
rather than by xanthine oxidase. However it is not yet known if the presence of this oxidase II is confined to the species studied, i.e. rat.

The nicotine $\Delta^{1'}(5')$ iminium ion has been shown to be a substrate for aldehyde oxidase. Interest concerning the oxidation of this compound arose when it was postulated that the second of two reaction steps involved in the biotransformation of (-)-nicotine to (-)-cotinine was mediated by aldehyde oxidase. Cotinine in man and most other species is the major metabolite of nicotine metabolism.  

![Chemical structure of nicotine and cotinine](image)

- **Nicotine**
- **Cotinine**

$py = 3$-pyridyl

The first reaction step seems to be hydroxylation catalysed by the cytochrome P-450 system and the product formed initially should thus be $5'$-hydroxynicotine (1b). In aqueous solution this pseudobase (1b) is in equilibrium with the cation (1a) and the iminium ion has in fact
been identified as a discrete intermediate in the oxidation of nicotine in vitro. The second reaction step was found by Hucker et al. to be mediated by a soluble enzyme which was suggested to be "an aldehyde oxidase". In 1972, Hill et al. showed that (1), generated in situ from nicotine, mouse liver microsomes, oxygen, and NADPH, could indeed be oxidised to cotinine by purified rabbit liver aldehyde oxidase.

The recent synthesis of the iminium ion has allowed further experiments to elucidate its role in cotinine formation. Gorrod and Hibberd demonstrated the conversion of (1) to cotinine by a hepatic cytoplasmic enzyme and identified the source of oxygen in cotinine as water. Brandänge and Lindblom found the ratio of the specific activities for N\(^1\)-methylnicotinamide and (1) to remain constant during purification of rabbit liver aldehyde oxidase. They concluded that aldehyde oxidase is the main, if not the sole enzyme mediating the oxidation of (1), and proposed from kinetic studies that the species (1a) rather than (1b) is the substrate. The \(K_m\) value obtained for nicotine \(\Delta^1(5')\) iminium ion of \(2 \times 10^{-6} \text{ M (pH 7.4)}\) shows this to be one of the best substrates known for aldehyde oxidase. These authors suggested the enzyme be renamed "iminium oxidase".

The biotransformation of azapetine has been studied in vitro with rat and rabbit hepatic subcellular fractions. Results suggest that the biotransformation to the lactam oxazapetine occurs probably via aldehyde oxidase in a very similar way to that of nicotine.

\[
\text{AZAPETINE} \quad \xrightarrow{1} \quad \text{IMINIUM ION} \quad \xrightarrow{2} \quad \text{OXAZAPETINE}
\]

1-NADPH dependent mixed function oxidase, 2-cytosolic enzyme (possibly aldehyde oxidase)
It has been suggested that other cyclic amine drugs, which are known to be oxidised to the corresponding lactam, may give rise to this type of iminium ion intermediate as a result of microsomal oxidation and that aldehyde oxidase may then play an important role in further oxidative transformation.

The few N-heterocyclic cations that have been studied with aldehyde oxidase have yielded some unusual and interesting results. Thus further research with this group of compounds is warranted.

1.3 Oxidising Substrate Specificity (Electron Acceptors)

Oxidising substrates react with the reduced enzyme by accepting electrons and hence may also be called electron acceptors. The term "oxidase" for both aldehyde oxidase and xanthine oxidase relates to their efficient use of oxygen as an electron acceptor. Xanthine dehydrogenases from avian sources use NAD\(^+\) and bacterial sources of this enzyme utilise ferredoxin more efficiently than oxygen. It has been suggested that in vivo some xanthine oxidase functions as the dehydrogenase and that purification processes cause the change in specificity for electron acceptor from NAD\(^+\) to oxygen. There has been no report of a dehydrogenase form of aldehyde oxidase.

Aldehyde oxidase and xanthine oxidase are able to utilise a wide range of electron acceptors including ferricyanide, dichlorophenolindophenol, cytochrome c, methylene blue and tetrazolinium salts. Although quinones such as menadione and p-benzoquinone also act as electron acceptors for xanthine oxidase; they are potent inhibitors of aldehyde oxidase. Phenazine methosulphate is unusual in that it can accept electrons under anaerobic conditions but under aerobic conditions acts as a reducing substrate with aldehyde oxidase. Certain nitro compounds can also function as electron acceptors for aldehyde oxidase and these compounds
compete effectively with oxygen for electrons. Aldehyde oxidase has recently also been shown to be capable of the reduction of sulphoxides.

1.4 Preparation

Recent methods for preparation of purified aldehyde oxidase are based on the procedure developed by Rajagopalan et al. in 1962, who obtained the enzyme free from haem contamination and which was stated to be approximately 90% pure (400 fold purification). Table 1.2 shows the procedure adopted by Felsted and Chaykin for the isolation of rabbit liver enzyme purified to 99% homogeneity.

<table>
<thead>
<tr>
<th>Step</th>
<th>Specific Activity (units/mg)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Homogenisation and heat treatment (55°C for 11 mins)</td>
<td>0.0162</td>
<td>100</td>
</tr>
<tr>
<td>2. (NH₄)₂SO₄ fractionation (0-50% sat.)</td>
<td>0.0558</td>
<td>98</td>
</tr>
<tr>
<td>3. Acetone fractionation (42-48%)</td>
<td>0.158</td>
<td>70</td>
</tr>
<tr>
<td>4. Calcium phosphate gel</td>
<td>0.577</td>
<td>42</td>
</tr>
<tr>
<td>5. DEAE-Sephadex chromatography</td>
<td>1.31</td>
<td>22</td>
</tr>
<tr>
<td>6. Preparative electrophoresis (Acrylamide gel)</td>
<td>1.89</td>
<td>15</td>
</tr>
</tbody>
</table>

Steps used by Rajagopalan et al., followed by aluminia gel absorption and dialysis. 

One unit of enzyme is that amount which causes conversion of 1 μmol of N⁷-methylnicotinamide to its pyridones per minute at 25°C.
This procedure resulted in a product which was stable for at least a month if stored in 3% sucrose at 4°C or alternatively, quick-frozen and stored at -70°C. The enzyme tends to polymerise on storage but this can be prevented or reversed by 0.005 M cysteine. Highly purified preparations have also been obtained from hog\textsuperscript{44} and \textit{D. melanogaster}\textsuperscript{69}.

Generally the method of isolation of aldehyde oxidase has depended upon which aspect of the enzyme investigators have been interested in. The 100,000 g supernatant fraction has often been used as a source of the enzyme where oxidation product investigation was the primary objective.\textsuperscript{29,70} The partially pure enzyme resulting from the ammonium sulphate fractionation step gives a reasonable compromise between stability, purity and activity and thus this preparation is often used as a readily available source of the enzyme for general work.\textsuperscript{29,39}

The most widely used method of assay of aldehyde oxidase employs N\textsuperscript{1}-methylnicotinamide as substrate and oxygen as the electron acceptor.\textsuperscript{58,68} Increase in optical density at 300 nm due to formation of product is used to measure the activity of the enzyme. A similar spectroscopic assay involving xanthine and oxygen is employed for xanthine oxidase.\textsuperscript{71}

1.5 Molecular Properties

Aldehyde oxidase contains molybdenum, FAD, and non-haem iron (as Fe/S clusters) in the ratio 1:1:4. The fully functional enzyme molecule is dimeric, being composed of two identical protein subunits, each possessing an active centre, \textit{i.e.} one enzyme molecule contains two atoms of molybdenum, two molecules of FAD and eight atoms of non-haem iron.\textsuperscript{1} Molecular weight determinations using disc gel electrophoresis and ultracentrifugal analysis gave 270,000 for hog liver enzyme and 260,000 for rabbit liver enzyme.\textsuperscript{44} Xanthine oxidase is similarly composed and has a molecular weight of 283,000.\textsuperscript{72}
The presence of Coenzyme Q10 had been suggested in earlier preparations of reasonably pure aldehyde oxidase. More recent workers involved in further purification of the enzyme did not mention the presence of this compound. In 1974, in a systematic analysis of fifty livers, Coenzyme Q10 was not detected and was concluded to have been a contaminant of previous aldehyde oxidase preparations.

The molybdenum centre is the site of reducing substrate interaction. The substrate, which binds to the active site, transfers a hydride ion (or two electrons and a proton) to the enzyme. The substrate is then attacked by a hydroxyl ion (from water) to yield the hydroxylated product. The molybdenum which has been reduced from Mo(VI) to Mo(IV) by electrons from the substrate, is reoxidised by distributing the electrons among the redox components of the enzyme (i.e., Fe/S, FAD groups). The final step is the oxidation of the reduced flavin chromophore with molecular oxygen. It has been pointed out that other electron acceptors can be used in place of oxygen, some of these accept electrons at a different site from which oxygen acts. All the prosthetic groups are reduced during the catalytic process, each therefore providing a possible site of electron egress.

1.5.1 Nature of the prosthetic groups

The flavin component was identified as FAD rather than FMN due to the increase in fluorescence which occurs when FAD is hydrolysed in acid. Non-haem iron was suggested to be linked to an acid labile sulphur because addition of sufficient HCl to the enzyme resulted in evolution of H₂S in an amount stoichiometric with the iron present.

1.5.1.1 U.v. spectra

Both FAD and the iron-sulphur chromophores contribute to the absorption spectrum of the enzyme. Branzoli and Massey were able to
prepare stable "deflavo" aldehyde oxidase, removing the flavin component by treating enzyme with calcium acetate and calcium chloride. The calculated difference spectrum between native and deflavo enzyme is typical of a flavoprotein. The spectrum of the deflavo enzyme is very similar to several iron-sulphur proteins such as plant ferredoxins. Thus it can be seen, from Figure 1.1, that while both FAD and iron-sulphur groups contribute to the absorption of the enzyme at 450 nm, the absorbance at 550 nm is due only to the iron-sulphur chromophore. The absorption spectra of purified aldehyde oxidase from both hog and rabbit liver are very similar to that reported for milk xanthine oxidase in both their native and deflavo forms. The $A_{450}/A_{550}$ ratio, usually close to 3, indicates an iron-sulphur flavin ratio of 4:1.

![Figure 1.1](image.png)

**Figure 1.1.** *Absorption spectra of aldehyde oxidase*

--- spectrum of native enzyme
--- spectrum of deflavo enzyme
*** calculated difference spectrum
1.5.1.2 E.p.r. spectra

Electron paramagnetic resonance (e.p.r.) studies have provided the greatest contribution to our understanding of the functioning of the prosthetic groups. The molybdenum, flavin and iron-sulphur centres all participate in the catalytic reaction.

\[
\begin{align*}
\text{Mo(VI)} & \rightarrow \text{Mo(IV)} \rightarrow \text{Mo(V)}^* \\
\text{FAD} & \rightarrow \text{FADH}^* \rightarrow \text{FADH}_2 \\
\text{Fe/S}_{\text{ox}} & \rightarrow \text{Fe/S}^*_{\text{red}}
\end{align*}
\]

Thus during the catalytic cycle each of the enzyme electron carriers can exist in a paramagnetic state (*) which can be monitored by e.p.r. The majority of e.p.r. studies on molybdenum hydroxylases relate to the commercially available xanthine oxidase rather than aldehyde oxidase. Experimental results are expressed in \( g \) values (determined by the ratio of the magnetic field \( H \) to the microwave frequency at which resonance occurs).

Recent e.p.r. studies have shown aldehyde oxidase to contain two distinct types of Fe/S centres, as does xanthine oxidase. The e.p.r. properties of the centres Fe/S I \( (g_{av} < 2) \) and Fe/S II \( (g_{av} > 2) \) are similar in both enzymes.

The paramagnetic semiquinone species of FAD can exist in different states of protonation.

\[
\begin{align*}
\text{H}_3\text{C} & \text{H}_3\text{C} \\
\text{N} & \text{N} \\
\dot{} & \dot{}
\end{align*}
\]

\[
\begin{align*}
\text{R} & \text{-H}^+ \\
\text{N} & \text{N} \\
\dot{} & \dot{}
\end{align*}
\]

Preferred tautomeric forms of the neutral and anionic radicals of the flavin semiquinone
Recently Barber et al. observed weak e.p.r. signals at \( g = 2.0036 \) with line width of 1.9 mT, this line width value is characteristic of neutral semiquinone radicals. Rajagopalan et al., some fourteen years earlier, obtained a lower value for the line width (1.6 mT) which was indicative of anionic semiquinone radicals; however the possibility that Coenzyme \( \text{Q}_{10} \) was present as a contaminant of their enzyme preparation could account for the smaller line width value obtained. Thus it would now appear that aldehyde oxidase forms a neutral semiquinone radical upon reduction. From line width measurements xanthine oxidase was also shown to form a neutral semiquinone.

E.p.r. spectra are particularly useful in studying the role of molybdenum which is not readily followed by any other technique, e.g. Bray and Swann showed that molybdenum makes little contribution to the electronic spectrum of the enzyme. A number of Mo(V) e.p.r. signals have been characterised for xanthine oxidase and given names. The signals relate to specific conditions and different configurations of the molybdenum centre.

Mo(V) signals from functional enzyme:

(i) Very Rapid, (ii) Rapid 1, (iii) Rapid 2, (iv) Inhibited (see Section 1.6.1.1).

Mo(V) signals from non-functional enzyme:

(v) Slow (see Section 1.5.4) and (vi) Desulpho Inhibited (or Resting II) (see Section 1.5.3.2.).

Signals (ii)-(vi) have also been observed with aldehyde oxidase. The Very Rapid signal (i) has only been observed on reduction of xanthine oxidase by xanthine or 1-methylxanthine and thus this signal is not generated with aldehyde oxidase as neither compounds
are substrates. Signals (iv), (v), (vi) will be discussed in relevant sections.

The current consensus of opinion is that substrate transfers two electrons to molybdenum reducing it from Mo(VI) to Mo(IV). Mo(IV) would then be partly reoxidised to yield the e.p.r. active Mo(V) species by intramolecular electron transfer to the iron-sulphur of flavin centres.\textsuperscript{83}

Rapid signals are characterised by hyperfine splitting of the Mo(V) signal by exchangeable protons.\textsuperscript{83} Rapid 2 spectra (iii) show coupling to two equivalent protons, whereas in Rapid 1 spectra (ii) one proton is strongly coupled to Mo(V) and the other only weakly coupled.\textsuperscript{83} In 1968 Rajagopalan et al.\textsuperscript{78} reported spectra obtained for aldehyde oxidase on reduction by substrate which bore only a moderate resemblance to those obtained with xanthine oxidase, with no real evidence for proton splitting ($g = 1.97$). Barber et al.\textsuperscript{77} reported difficulty in measuring such signals because of interference by signals due to non-functional enzyme in their preparations. Bray et al.\textsuperscript{84} apparently did not have this problem but found that spectra obtained using phthalazine, salicylaldehyde or purine again showed only a general resemblance to the typical Rapid 1 spectra. Clearer results were obtained when aldehyde oxidase had been pretreated with 2-methylquinoline prior to its reduction with phthalazine. Stubley\textsuperscript{86} reported that 2-methylquinoline is oxidised by the enzyme to an inhibitor thought to be 2-hydroxymethylquinoline. The spectra so obtained were found to be a mixture of Rapid 1 and 2 signals which were resolved by difference spectra techniques. The Rapid 1 signal was found to be similar for both enzymes. The presence of exchangeable protons was confirmed by loss of the doublet feature on reduction of the enzyme in $^2\text{H}_2\text{O}$.

It should be possible to obtain structural information about the active centre of the enzymes by comparing their e.p.r. spectra with those of Mo(V) compounds of known structure. However, to date, there are few
well-characterised mono-nuclear Mo(V) compounds so this is a difficult task. What is clear from the remarkable similarities in the molybdenum spectra of all the molybdenum hydroxylases is that the environments of the metal in these enzymes must be essentially identical. Bray et al. suggested that the striking difference in substrate specificity that exists between aldehyde oxidase and the other enzymes must therefore be due primarily to structural differences in regions of the active centres concerned solely with substrate binding, rather than to differences in the catalytically important molybdenum sites.

Measurement of the strength of magnetic interaction between the prosthetic centres can yield information about their spatial relationships. The Mo(V) e.p.r. spectra obtained from deflavo aldehyde oxidase and xanthine oxidase are almost identical to those obtained from the native enzymes, indicating that the metal is not in the immediate vicinity of the flavin in the enzyme. Strong magnetic interactions exist between Mo(V) and Fe/S I for aldehyde oxidase and xanthine oxidase but no Mo(V)-Fe/S II interaction has been detected for either enzyme, suggesting that for both enzymes the Fe/S I group (but not the Fe/S II group) is in close proximity to the molybdenum centre. However differences were observed between these two enzymes, for example, no magnetic interaction between the Fe/S I and Fe/S II centres was detected for aldehyde oxidase, whereas such interaction was readily observed in xanthine oxidase. Furthermore, the Fe/S-FAD interactions observed for xanthine oxidase were much stronger than those for aldehyde oxidase. These results suggest that arrangement of the prosthetic groups must differ in the two enzymes, although the molybdenum environment is similar. Distances between the various groups can be calculated from magnetic interaction measurements. Figure 1.2 shows the distances between prosthetic groups in a xanthine oxidase.
subunit obtained by Barber et al.\textsuperscript{94} in 1982.

**Figure 1.2.** Proposed model for the arrangement of the prosthetic groups in xanthine oxidase

![Diagram of prosthetic groups](image)

**1.5.2 The molybdenum cofactor**

The molybdenum atom plays a key role in the functioning of the enzymes containing this element (e.g. molybdenum hydroxylases, nitrate reductase and nitrogenase) and there has been considerable discussion concerning the presence of a common molybdenum cofactor. In 1970, a mutant (nit-1) of *Neurospora crassa* which lacked nitrate reductase activity was reported.\textsuperscript{95} Acidified samples of molybdenum hydroxylases, including aldehyde oxidase, when added to an extract of nit-1 produced an active nitrate reductase. It was proposed that all the molybdenum containing enzymes possess a common molybdenum cofactor which is released upon acid treatment. This proposal has been supported by more recent results,\textsuperscript{96–99} except in the case of nitrogenase which has a different cofactor.\textsuperscript{100} The molybdenum cofactor is thought to be non-covalently attached to protein,\textsuperscript{99} and appears to be a small molecule with a molecular weight of ca. 1,000.\textsuperscript{101} The free cofactor is very labile and consequently
purification and characterisation are difficult. The loss of molybdenum cofactor activity (as measured in an assay with nit-1) is accompanied by increasing fluorescence. Recent workers have been concerned with the elucidation of the structure of the stable oxidised forms of the cofactor which are inactive. The inactive forms of the cofactor have been identified as 6-substituted pterins by fluorescence spectroscopy. The active cofactor is presumably composed of molybdenum and a reduced form of the pterin. Other cofactors which contain pterin structures are generally biologically active in the fully reduced form, which is non-fluorescent and oxygen labile. The pterin has also been shown to have a sulphur bound at position 7, which has been suggested to bind the pterin to molybdenum. However despite considerable effort the precise structure and role of this cofactor remains unclear.

1.5.3 Naturally occurring modified forms of aldehyde oxidase

Table 1.3 summarises some of the molecular properties of aldehyde oxidase and related enzymes.

Table 1.3. Molecular properties of molybdenum-containing enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Source</th>
<th>Molecular Weight</th>
<th>Analysis (mole/mole enzyme)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fe</td>
</tr>
<tr>
<td>Aldehyde Oxidase</td>
<td>hog</td>
<td>270,000</td>
<td>8.2</td>
</tr>
<tr>
<td>Aldehyde Oxidase</td>
<td>rabbit</td>
<td>260,000</td>
<td>7.92</td>
</tr>
<tr>
<td>Xanthine Oxidase</td>
<td>milk</td>
<td>283,000</td>
<td>8</td>
</tr>
<tr>
<td>Xanthine Dehydrogenase</td>
<td>chicken</td>
<td>300,000</td>
<td>7.98</td>
</tr>
<tr>
<td>Xanthine Dehydrogenase</td>
<td>turkey</td>
<td>280,000</td>
<td>7.92</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Value from Massey \textit{et al.}\textsuperscript{102}

\textsuperscript{b} Value from Hart \textit{et al.}\textsuperscript{71}
The molybdenum hydroxylases are similar in physical properties despite the considerable variation in catalytic activity between them. All fully functional enzyme molecules are protein dimers containing two molybdenum atoms, two flavin molecules and eight non-haem iron atoms complexed with acid-labile sulphur ligands. In addition, the presence of a specific cyanolysable sulphur atom located at the molybdenum centre has been identified as being essential for catalytic activity (see below). Non-functional forms of aldehyde oxidase have been identified in enzyme preparations of which the demolybdo and desulpho are the best characterised.

1.5.3.1 Demolybdo enzyme

Purified preparations of aldehyde oxidase from a number of rabbits were reported by Felsted et al.\textsuperscript{44} to have 0.66 to 1.4 g atoms of molybdenum per mole of enzyme; which is lower than the generally accepted molar content of two. These workers attributed the low values obtained to partial loss of metal during purification, as the molar content of the other prosthetic groups (Fe/S and FAD) was very similar to that expected, \textit{i.e.} 8 and 2 respectively (see Table 1.3). However Bray\textsuperscript{103} suggested that, by analogy with xanthine oxidase, the preparations probably contained an inactive demolybdo form which occurs naturally rather than being produced as an artefact during preparations. Hart et al.\textsuperscript{71} found the molar content of molybdenum to be less than 2, for preparations of milk xanthine oxidase and this lower value was attributed to the presence of some demolybdo form, the relative amount of which did not alter during enzyme preparation. However Massey et al.\textsuperscript{102} did not find any demolybdo enzyme in their preparations of xanthine oxidase (\textit{i.e.} Mo molar content of 2). Administration of tungsten to rats was shown to decrease hepatic xanthine oxidase activity by preventing incorporation of molybdenum into the enzyme and the demolybdo enzyme so produced was isolated.\textsuperscript{104,105} From these results it was proposed
that a demolybdo form is secreted along with the active form but that the relative amounts are nutritionally determined.

1.5.3.2 Desulpho enzyme

Aldehyde oxidase has been shown to be inactivated by cyanide.\(^3\) This inactivation is significantly retarded by uncharged substrates, but not by charged substrates, such as \(N^1\)-methylnicotinamide.\(^3\) When aldehyde oxidase\(^106\) or xanthine oxidase\(^107\) was treated with cyanide, thiocyanate was detected, indicating cyanide inactivation results from abstraction of sulphur. Both enzymes can be readily reactivated using sulphide in the presence of reducing agents, e.g. dithionite.\(^108\)

Branzoli and Massey identified a non-functional form of enzyme in their preparations of aldehyde oxidase which lacked the cyanolysable sulphur.\(^106\) This naturally-occurring non-functional "desulpho" enzyme and cyanide inactivated enzyme exhibit very similar properties and are thought to be identical,\(^109\) e.g. naturally occurring desulpho enzyme can also be reconstituted on incubation with sulphide and dithionite.\(^108\) Application of the resulphuration procedure to extracts of \textit{Drosophila ma-1} flies which show deficiencies in xanthine dehydrogenase and aldehyde oxidase led to emergence of enzyme activity. Thus it was suggested that the incorporation of cyanolysable sulphur into molybdenum hydroxylases is genetically controlled.\(^109\) Desulpho enzyme has been separated from active sulpho enzyme in preparations of xanthine oxidase by affinity chromatography.\(^107,110\)

The content of fully functional molecules in preparations having the full complement of prosthetic groups can be determined by measuring the amount of thiocyanate released on inactivation by cyanide.

Treatment of desulpho xanthine oxidase with ethylene glycol produces a characteristic Mo(V) e.p.r. spectrum named \textit{Resting II} or \textit{Desulpho Inhibited}.\(^117\) This signal is similarly obtained with aldehyde oxidase.\(^77,84\)
Desulpho Inhibited signal can also be observed in native aldehyde oxidase in the absence of reducing agent.\textsuperscript{77,78} It was suggested that this signal arises from interaction of the naturally occurring desulpho form of the enzyme with contaminating aldehydes or alcohols during enzyme preparation.\textsuperscript{117} Thus it would appear that desulpho enzyme, although devoid of catalytic activity, still has the ability to bind substrates at the molybdenum centre.\textsuperscript{111}

1.5.4 **Identity of the cyanolysable sulphur**

The position of the cyanolysable sulphur atom at, or near, the molybdenum is now accepted but its precise nature and bonding remains unclear. Various workers have suggested that the sulphur atom is part of a cysteine group,\textsuperscript{112} a persulphide\textsuperscript{113} or is a molybdenum sulphide.\textsuperscript{114} Recent work favours the molybdenum sulphide proposal. X-ray data concerning xanthine oxidase gave a Mo-S bond distance of 0.225 nm\textsuperscript{115} which is shorter than expected for a persulphide or cysteine linkage. However in the desulpho enzyme this terminal sulphur atom appears to be replaced by an oxygen atom.\textsuperscript{115} These observations are in accord with the proposal of Gutteridge \textit{et al.} that the cyanide removes the terminal sulphur (which is then replaced by oxygen\textsuperscript{114}), \textit{viz}.

\[
(\text{Enzyme})-\text{Mo}=S + \text{CN}^- + \text{H}_2\text{O} \longrightarrow (\text{Enzyme})-\text{Mo}=O + \text{CNS}^- + 2e + 2\text{H}^+
\]

It was also proposed that reduction of both active and desulpho enzyme was accompanied by protonation.\textsuperscript{114}

\[
\begin{align*}
\text{FUNCTIONAL} & \quad (\text{Enzyme})-\text{Mo}=S + \pm\text{H}^+ \longrightarrow (\text{Enzyme})-\text{Mo}^+-\text{SH} & (\text{Eqn. a}) \\
\text{DESULPHO} & \quad (\text{Enzyme})-\text{Mo}=O + \pm\text{H}^+ \longrightarrow (\text{Enzyme})-\text{Mo}^+-\text{OH} & (\text{Eqn. b})
\end{align*}
\]

X-ray data obtained for xanthine dehydrogenase\textsuperscript{116} was interpreted by Spence\textsuperscript{87} as shown below:
Only the oxidised forms of the enzymes are inactivated by cyanide. By analogy to the chemical situation, $S^0$ is susceptible to cyanide attack ($CN^- + S^0 \rightarrow SCN^-$) whereas sulphides ($\sim SH$) are inert. Reactivation of the desulpho enzyme by sulphide is facilitated by the presence of reducing agents, e.g. dithionite. The effect of dithionite is suggested to be due to conversion of the additional oxo group in the desulpho enzyme to Mo–OH; this OH group is labile and thus easily replaced by sulphide to reactivate the enzyme.

$$Mo(IV)OH + SH^- \rightarrow Mo(IV)SH + OH^-$$

The Slow $Mo(V)$ e.p.r. signal obtained from desulpho enzyme has been observed for aldehyde oxidase and is said to resemble that obtained for xanthine oxidase. The Slow signal, like the Rapid signal obtained from functional enzyme is coupled to protons. $pK_a$ measurements (with xanthine oxidase) of the strongly coupled proton show that the desulpho enzyme has a $pK_a$ of 10 and the functional enzyme has a $pK_a$ of 8. This also supports the Gutteridge et al. proposal (Equations a and b above) as
protons bound to sulphur are found to be more acidic than those bound oxygen. Desulpho enzyme is still able to bind substrate but its general lack of catalytic activity is suggested to be due to its lesser ability to accept protons compared to functional enzyme. Thus it would appear that the cyanolysable sulphur atom is intimately involved with molybdenum and the catalytic process.

1.6 Sites of Substrate Interaction

1.6.1 Reducing substrates

It is now generally accepted that reducing substrates such as purine bind at the molybdenum centre. E.p.r. studies and the use of chemical inhibitors and modifiers of the enzyme have led to this deduction. Thus these substrates influence the Mo(V) e.p.r. spectra directly and specifically but have no influence on signals from other chromophores. The substrate is considered to transfer two electrons to molybdenum reducing it from Mo(VI) to Mo(IV). The Mo(IV) species is then partly reoxidised to yield the e.p.r. active Mo(V) species by intramolecular electron transfer to the iron-sulphur or flavin centres. Interaction of 8-[13C]-xanthine (i.e. labelled at the position of hydroxylation) with xanthine oxidase gives rise to a Very Rapid molybdenum(V) e.p.r. signal which shows 13C coupling and thus clearly demonstrates the close association of the substrate xanthine with the molybdenum centre. Demolybo enzyme is inactive towards reducing substrates and, as discussed in the last section, removal of cyanolysable sulphur from the molybdenum centres prevents reduction of the enzyme.

1.6.1.1 Active site directed inhibitors

Further evidence concerning substrate binding and the role of molybdenum has come from studying active site directed inhibitors.
Xanthine oxidase is inhibited by allopurinol; inhibition results from complex formation of the reduced \([\text{Mo(IV)}]\) enzyme by the product of allopurinol oxidation, alloxanthine.\(^27\) By briefly exposing the inhibited enzyme to air, a new, broader Mo(V) e.p.r. signal was generated which was attributed to a one electron oxidised product, a Mo(V) complex with alloxanthine. This signal showed coupling to \(^{14}\text{N}\) and indicates binding of the inhibitor to the molybdenum centre at the N-8 position of alloxanthine.\(^{119}\) In contrast, oxidation of allopurinol by aldehyde oxidase does not result in inhibition.\(^{26}\)

Xanthine oxidase and aldehyde oxidase are inhibited by arsenite, but whereas inhibition of the former is weak and non-competitive,\(^120\) inhibition of aldehyde oxidase is extremely rapid and competitive in nature \((K_i = 6.3 \times 10^{-6} \text{ M})\).\(^3,121\) Preincubation with cyanide prevents subsequent reactions with arsenite but reverse protection is only partial.\(^{121}\) In 1983 Mo(V) e.p.r. studies showed nuclear hyperfine coupling of the \(^{75}\text{As}\) nucleus to molybdenum with aldehyde oxidase, xanthine oxidase and xanthine dehydrogenase.\(^{122}\) Arsenite thus appears to be closely bound to the molybdenum centre as suggested by earlier inhibition studies.\(^{121}\) Reduction potential measurements reveal that arsenite greatly stabilises the Mo(V) oxidation state of these enzymes, binding by a factor of ten more tightly to Mo(V) than to either Mo(IV) or Mo(VI).\(^{122}\) Thus the finding that arsenite, which inhibits substrate oxidation, binds at the molybdenum centre provides further evidence that reducing substrates must also bind at this centre.

Both xanthine oxidase and aldehyde oxidase undergo progressive inactivation when assayed in the presence of methanol.\(^3,121\) Methanol inactivation gives rise to a characteristic Mo(V) e.p.r. signal called the Inhibited signal which is air-stable.\(^{123}\) Studies with xanthine oxidase have shown this signal to be coupled to a single proton derived from
methanol which is not exchangeable; the same signal was obtained using formaldehyde.\textsuperscript{123} It was suggested that methanol inactivation results from its conversion at the active site to formaldehyde with subsequent formylation (-CHO) of the molybdenum centre. Formaldehyde is a very poor substrate for aldehyde oxidase\textsuperscript{9} and causes denaturation of hog enzyme.\textsuperscript{2}

The Mo(V) e.p.r. \textit{Inhibited} signal has been observed with aldehyde oxidase from both methanol\textsuperscript{77,78} and formaldehyde\textsuperscript{84} indicating the site of interaction of these compounds to be the molybdenum centre.

Treatment of functional xanthine oxidase with ethylene glycol produced a Mo(V) e.p.r. signal similar to that obtained with methanol or formaldehyde, but it lacked any proton hyperfine splitting.\textsuperscript{124} If ethylene glycol was oxidised at the molybdenum centre in an analogous way to methanol then this would result in the group, -COCH\textsubscript{2}OH, complexing with the enzyme and consequently this would not be expected to show any strong proton coupling to molybdenum(V) because it lacks an \(\alpha\)-hydrogen. Ethylene glycol has actually been shown to undergo xanthine oxidase catalysed oxidation to glycolic acid and oxalic acid, although this occurs extremely slowly\textsuperscript{125} (turnover is \(10^5-10^6\) slower than with xanthine).

Thus it can be concluded from the work on reducing substrates and active site directed inhibitors that both aldehydes and heterocyclic compounds react at the molybdenum centre of the molybdenum hydroxylases.

1.6.2 \textbf{Sites of interaction of the oxidising substrates (electron acceptors)}

Oxidising substrates accept electrons from the reduced enzyme and thus become reduced while the enzyme is reoxidised. All the prosthetic groups are involved in the catalytic process, each therefore providing a possible site of electron egress. Determination of the site(s) of interaction of the various oxidising substrates (listed on page 15) has been achieved by monitoring the effects of specific inhibitors and chemical modifiers of the prosthetic groups on the activities of these substrates.
Deflavo forms of both aldehyde oxidase and xanthine oxidase are devoid of catalytic activity when using oxygen as the electron acceptor; however activity towards oxygen can be restored on addition of FAD. It is the flavin component which is therefore considered to be the site of interaction with oxygen. Both enzymes reduce oxygen by a combination of one and two electron transfer steps.\textsuperscript{126,127}

\[
\text{FADH}_2 + 2\text{O}_2 \rightarrow \text{FAD} + 2\text{H}^+ + 2\text{O}_2^- \\
\text{FADH}_2 + \text{O}_2 \rightarrow \text{FAD} + \text{H}_2\text{O}_2
\]

Earlier workers had assumed that reduction occurred only by 2-electron transfer producing hydrogen peroxide, but subsequently the presence of the superoxide free radical was detected by e.p.r. spectroscopy.\textsuperscript{128} This superoxide ion, which arises from a 1-electron reduction process, is responsible for the chemiluminescence observed during turnover of the enzymes in the presence of oxygen.\textsuperscript{60}

Cytochrome c can only act as an electron acceptor for aldehyde oxidase in the presence of oxygen and is inhibited by superoxide dismutase which catalyses the reaction\textsuperscript{129}

\[
2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{O}_2 + \text{H}_2\text{O}_2
\]

From this it can be concluded that cytochrome c reduction by aldehyde oxidase is mediated exclusively by the superoxide radical.

\[
\text{Cytochrome c}^{3+} + \text{O}_2^- \rightarrow \text{Cytochrome c}^{2+} + \text{O}_2
\]

With xanthine oxidase cytochrome c reduction proceeds mainly by superoxide ions but it can also be reduced anaerobically, i.e. there is some direct electron transfer from reduced enzyme to cytochrome c.\textsuperscript{130}

Aldehyde oxidase can reduce nitroblue tetrazolium (NBT) by two independent mechanisms: either by direct interaction with the flavin
chromophore or indirectly via superoxide ions. Reduction occurs most readily under anaerobic conditions, but workers have also observed some NBT activity under aerobic conditions which was inhibited by superoxide dismutase. Deflavo enzyme was shown to be devoid of activity towards NBT.

Using rabbit liver aldehyde oxidase Rajagopalan and Handler studied the effect of various inhibitors upon electron acceptor activity. Modification of the molybdenum centre, e.g. via cyanide and mercuribenzoate decreased the oxidation of N\textsuperscript{1}-methylnicotinamide regardless of acceptor used. A second class of inhibitors investigated included: amytal, oestradiol, menadione and triton X-100 (see Table 1.4, page 35). These compounds inhibit the electron transport system of aldehyde oxidase but not of xanthine oxidase. In fact menadione, like other quinones, acts as an electron acceptor for xanthine oxidase. Dichlorophenolindophenol (DCIP) and ferricyanide are both capable of accepting electrons from deflavo enzyme and so their interaction site must be either at molybdenum or the iron-sulphur centres. DCIP reduction is unaffected by this class of inhibitor whereas ferricyanide is inhibited by all of them; thus these two oxidising substrates must act at different sites: the former at molybdenum and the latter at the iron-sulphur centres. DCIP also competitively inhibits the xanthine oxidase catalysed oxidation of both xanthine and acetaldehyde. Gurtoo and Johns detected a complex between xanthine oxidase and DCIP spectrophotometrically and concluded that the site of binding was the molybdenum centre.

The results of these inhibition studies were originally interpreted in terms of a linear sequence of electron transfer from the substrate through various redox groups of the enzyme with oxygen acting as the terminal entity of this sequence (i.e. Substrate → Mo → Fe/S → FAD → O\textsubscript{2}). However it is now generally accepted that the redox centres are
Table 1.4. Effect of inhibitors on rabbit liver aldehyde oxidase

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Menadione</td>
<td>$2.0 \times 10^{-7}$ M</td>
<td>85&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Menadione diphosphate</td>
<td>$1.0 \times 10^{-4}$ M</td>
<td>0</td>
</tr>
<tr>
<td>Benzoquinone</td>
<td>$1.0 \times 10^{-5}$ M</td>
<td>100</td>
</tr>
<tr>
<td>Hydroquinone</td>
<td>$1.0 \times 10^{-4}$ M</td>
<td>40</td>
</tr>
<tr>
<td>Coenzyme Q&lt;sub&gt;10&lt;/sub&gt;</td>
<td>$1.0 \times 10^{-5}$ M</td>
<td>0</td>
</tr>
<tr>
<td>Amytal&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>$1.0 \times 10^{-3}$ M</td>
<td>69&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Antimycin A&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>$2.0 \times 10^{-6}$ M</td>
<td>62&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Estradiol&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>$2.0 \times 10^{-6}$ M</td>
<td>62&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Progesterone&lt;sup&gt;c&lt;/sup&gt;</td>
<td>$6.7 \times 10^{-5}$ M</td>
<td>45&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2,4-Dinitrophenol</td>
<td>$3.3 \times 10^{-4}$ M</td>
<td>50&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dicoumarol</td>
<td>$1.0 \times 10^{-4}$ M</td>
<td>36&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Triton X-100&lt;sup&gt;d&lt;/sup&gt;</td>
<td>$2.0 \times 10^{-4}$ Z</td>
<td>61</td>
</tr>
<tr>
<td>Sodium cholate</td>
<td>$1.0 \times 10^{-4}$ Z</td>
<td>0</td>
</tr>
<tr>
<td>Quinacrine</td>
<td>$1.0 \times 10^{-4}$ Z</td>
<td>83</td>
</tr>
</tbody>
</table>

<sup>a</sup> After 5 minutes  
<sup>b</sup> After 10 minutes  
<sup>c</sup> Also inhibitors of mitochondrial electron transport<sup>133</sup>  
<sup>d</sup> Further used by Rajagopalan and Handler<sup>64</sup> to study effect upon electron acceptor specificities.
Figure 1.3. Proposed sites of interaction of electron acceptors with aldehyde oxidase
arranged in a non-linear fashion and that reversible electron transfer occurs between the centres.

This information is collated in Figure 1.3 to show the proposed sites of interaction of the various oxidising substrates (electron acceptors) with aldehyde oxidase.

1.7 Enzyme Reduction and Reoxidation

Internal electron transfer between the prosthetic groups has been found to be much more rapid than turnover of substrate.\(^{128,134}\) Therefore the oxidation states of the various enzyme centres during the catalytic cycle should be determined by their relative reduction potentials.

Olson et al.\(^ {134}\) calculated sets of relative potentials for xanthine oxidase while recent workers have determined absolute potentials for the various centres of several molybdenum hydroxylases.\(^ {77,92,135}\)

Table 1.5. Midpoint reduction potentials for prosthetic groups of aldehyde oxidase, milk xanthine oxidase and chicken liver xanthine dehydrogenase\(^ {77}\)

<table>
<thead>
<tr>
<th>Prosthetic Group</th>
<th>Aldehyde Oxidase</th>
<th>Xanthine Oxidase</th>
<th>Xanthine Dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAD/FADH(^+)</td>
<td>-258</td>
<td>-310</td>
<td>-345</td>
</tr>
<tr>
<td>FADH(^+)/FADH(_2)</td>
<td>-212</td>
<td>-220</td>
<td>-377</td>
</tr>
<tr>
<td>Fe/S I (ox/red)</td>
<td>-207</td>
<td>-280</td>
<td>-280</td>
</tr>
<tr>
<td>Fe/S II (ox/red)</td>
<td>-310</td>
<td>-245</td>
<td>-275</td>
</tr>
<tr>
<td>Mo(VI)/Mo(V) rapid(^a)</td>
<td>-359</td>
<td>-355</td>
<td>-357</td>
</tr>
<tr>
<td>Mo(V) rapid/Mo(IV)</td>
<td>-351</td>
<td>-335</td>
<td>-337</td>
</tr>
<tr>
<td>Mo(VI)/Mo(V) slow(^b)</td>
<td>-439</td>
<td>-354</td>
<td>-397</td>
</tr>
<tr>
<td>Mo(V) slow/Mo(IV)</td>
<td>-401</td>
<td>-386</td>
<td>-433</td>
</tr>
</tbody>
</table>

\(a\) Potentials involving Mo(V) Rapid species obtained with native enzymes

\(b\) Potentials involving Mo(V) Slow species obtained with cyanide treated (desulpho) enzymes

\(c\) pH 7.8
The flavin centre is much more easily reduced in aldehyde oxidase than either of the xanthine oxidising enzymes. This was deduced from the results presented in Table 1.5, the reduction potential for FAD/FADH* being much more positive in aldehyde oxidase than in xanthine oxidase or xanthine dehydrogenase. The order of reduction of the two iron-sulphur centres in aldehyde oxidase as concluded from midpoint potentials, is Fe/S I (-207 mV) followed by Fe/S II (-310 mV) whereas the reverse is true (i.e. Fe/S II is reduced first) for xanthine oxidase and xanthine dehydrogenase.

The midpoint reduction potentials of molybdenum, flavin, and iron-sulphur centres of xanthine oxidase were found to be dependent on pH. This suggested that electron uptake by a centre is accompanied by protonation.

The methods employed for reduction potential measurements have mainly involved poising the system at a given overall reduction state at room temperature and then determining electron distribution by e.p.r. at 25-120 K. This assumes that electron distributions measured at these low temperatures are the same as those at room temperature. Some recent work has been concerned with testing this assumption. Spence et al. obtained reduction potential values for xanthine oxidase at room temperature using microcoulometry. Porras and Palmer used circular dichroism to monitor the iron-sulphur centres and room temperature e.p.r. to monitor flavin and molybdenum centres. Both sets of workers showed the midpoint potentials to be temperature dependent, although the extent of this temperature effect varied with conditions and the centre under consideration. However Porras and Palmer remarked that the differences were not so substantial as to radically alter the current model of electron transfer proposed for xanthine oxidase.

If each Fe/S centre takes up one electron and the FAD and
molybdenum centres two each, then full reduction of either aldehyde oxidase or xanthine oxidase should require six electrons per enzyme subunit. Working with xanthine oxidase Edmondson et al.\textsuperscript{107} and Olson et al.\textsuperscript{128,134} observed close to this stoichiometry in reductive titrations with dithionite, when the end point was determined by measuring absorbance or e.p.r. changes in the enzyme itself. However, when they followed the disappearance of dithionite, by means of its absorbance at 315 nm, these workers found consistent uptake of eight electrons per enzyme subunit. Hille and Massey\textsuperscript{138} reported the presence of a disulphide bond in xanthine oxidase which could be reduced to account for the uptake of the two extra electrons. The disulphide bond was not thought to play a role in the catalytic reactions of the enzyme as xanthine was not capable of reducing this centre.

A mechanism of enzyme reoxidation has been proposed only for xanthine oxidase. Olson et al.\textsuperscript{134} suggested a scheme (see Figure 1.4)

\begin{center}
\includegraphics[width=0.8\textwidth]{reoxidation.png}
\end{center}

\textbf{Figure 1.4.} \textit{Suggested sequence of reoxidation of xanthine oxidase} in which this reoxidation occurs by way of a combination of one and two electron transfer steps to give both hydrogen peroxide and superoxide as products (also known to be products of aldehyde oxidase reoxidation). \(XO(n)\) represents a reduced enzyme species containing \(n\) electrons. Initially oxygen binds rapidly to the fully reduced flavin and an electron is transferred to generate a "flavin semiquinone-superoxide" complex. When the enzyme contains more than two electrons, fully reduced flavin (FADH\textsubscript{2}) is regenerated by intramolecular transfer from the reduced iron-
sulphur centre and molybdenum. The rate of the latter process is greater than the rate of $O_2^-$ diffusion out of the active site, the second electron being transferred to produce $H_2O_2$. This two electron reoxidation process occurs until $XO(2)$ is produced. Oxygen again binds rapidly but in the resulting complex, fully reduced flavin cannot be regenerated and rapid production of $O_2^-$ occurs. The production of $O_2^-$ from reaction of the one electron reduced state of the enzyme ($XO(1)$) is slow corresponding to the spectrophotometrically observed slow phase in reoxidation of the enzyme.

When the enzyme is fully reduced ($n = 6$) the major product will be $H_2O_2$ but at high oxygen and low xanthine concentration when the enzyme is cycling principally between 2-electron reduced ($XO(2)$) and oxidised enzyme ($XO(0)$), the primary product is $O_2^-$.  

1.8 **Mechanism of Hydroxylation**

It is now generally accepted that oxidation catalysed by the molybdenum hydroxylases is in fact a hydroxylation reaction and that the $OH^-$ ion is derived from water. However despite considerable discussion the precise nature of this process is still not clearly resolved. A few of the more recent hypotheses are briefly discussed below.

Rajagopalan and Handler \(^3\) proposed that hydroxylation takes place by concurrent removal of a hydride ion from the substrate with replacement by a hydroxyl ion from the medium. This reaction would be facilitated if a group on the enzyme acted as a Lewis acid, ligating the nitrogen adjacent to the carbon to be hydroxylated. Requirement for a Lewis acid is obviated for those substrates bearing a quaternary nitrogen, as the positively charged nitrogen withdraws electrons from the adjacent carbon thereby facilitating attack by a hydroxyl ion. The Lewis acid was suggested to be molybdenum. If this were the case, the interesting inference would be
that molybdenum is not involved in the initial binding of quaternised substrates to the enzyme. The mode of binding of quaternary compounds has since been neglected because generally ideas related to hydroxylation mechanism have centred on xanthine oxidase, for which these compounds are not good substrates.

In view of the known tendency of ligands of molybdenum to protonate more readily when the metal is in the lower oxidation states, Stiefel\textsuperscript{139} hypothesised that the role of the metal in these enzymes is to act as an electron acceptor, while a ligand simultaneously acts as the proton acceptor (\textit{i.e.} abstraction of 2e and H\textsuperscript{+}, rather than a H\textsuperscript{-}, from the substrate). This concept was also adopted by Olson\textsuperscript{134} and more recently by Bray\textsuperscript{140} in their proposed mechanisms (see below).

Olson \textit{et al.}\textsuperscript{134} in 1974 suggested the following scheme:

Following binding of the substrate to the active site, attack of a persulphide group causes splitting of the C\textsubscript{8}-H bond. This is accompanied by the donation of 2 electrons to Mo(VI). The rate of this process was regarded as being fast and dependent on the fraction of Mo(VI) present.
The C₈ proton is taken up by a group liganded to the metal, shown here as nitrogen. The final step involves attack by a water molecule which hydrolyses the persulphide-xanthine linkage causing the release of product.

Subsequently Bray et al.¹⁴⁰ pointed out that Olson's model does not fully account for some of the more recently obtained e.p.r. data. For example, the absence of nitrogen hyperfine splitting in the Mo(V) e.p.r. signal casts doubt on the proton accepting group being nitrogen; in fact evidence is more consistent with a sulphur atom being the proton acceptor (see Section 1.5.4). The concept of the N-9 atom of xanthine initially forming a ligand with molybdenum, followed by attack on the C₈ atom by a nucleophilic group at the active centre was still retained in Bray's mechanism. However the nucleophile (X⁻) was not specified as a persulphide group.

Splitting of the C₈-H bond with concerted proton transfer to the Mo=S group giving Mo-SH and transfer of 2 electrons to Mo(VI) was then proposed to occur. The nucleophile is ultimately replaced by an OH⁻ ion releasing the hydroxylated substrate.

The latter two models described are based on the interaction of xanthine oxidase with xanthine; how far can these be applied to the
mechanism of action of aldehyde oxidase? From Mo(V) e.p.r. studies it appears that the ligands and overall coordination geometries of the catalytically important molybdenum are the same in both these enzymes. Differences in substrate specificity probably result from structural variations at the substrate binding site of the enzymes. However it has been suggested that the overall mechanism of action may be regarded as being essentially similar in all the molybdenum hydroxylases.

AIMS AND SCOPE OF THE PRESENT INVESTIGATION

Although a number of N-heterocyclic cations are known to be good substrates for aldehyde oxidase, e.g. N-ethylquinolinium, there has not been a systematic investigation of this group of compounds. The N-heterocyclic cation N\textsuperscript{1}-methyl nicotinamide is apparently unique in that it yields two oxidation products (a 2-pyridone and a 4-pyridone) simultaneously with this enzyme. The formation of the 4-pyridone had been overlooked for a number of years for two main reasons. Firstly, aldehyde oxidase is usually prepared from rabbit liver. With enzyme from this source the major product is the 2-pyridone, the 4 isomer representing only a very small proportion of the total oxidation products. Secondly, the main analytical technique used to identify the oxidation product was ultraviolet spectroscopy which does not distinguish between the 2- and 4-pyridone. It has been suggested that these two oxidation products result from substrate binding at the enzyme active site in two different orientations. Many of the studies (e.g. e.p.r.) which identified the molybdenum centre of aldehyde oxidase as the site of substrate interaction employed N\textsuperscript{1}-methyl nicotinamide for this purpose. Recent proposals for the mechanism of hydroxylation, based on a non-quaternary compound \textit{vida supra}, involve the substrate binding \textit{via} its lone pair of electrons on nitrogen to the molybdenum centre with subsequent hydroxylation of the adjacent carbon. However, N-heterocyclic
cations have no lone pair of electrons on nitrogen available to donate to molybdenum, and in addition they often possess a bulky group attached to nitrogen.

Only in an earlier mechanism proposed by Rajagopalan and Handler\textsuperscript{3} had binding of N-heterocyclic cations been considered and these workers suggested it must be different from that of uncharged substrates (see page 40). From these interesting observations it would appear that the interaction of N-heterocyclic cations with aldehyde oxidase warrants further investigation.

Aza-heterocycles, e.g. quinoline, are good substrates for aldehyde oxidase and their oxidation with this enzyme has been well documented.\textsuperscript{6,21,29,31} It is therefore purposed to investigate the specificity of aldehyde oxidase \textit{in vitro} towards a number of quaternised aza-heterocycles, some of which are shown on page 47. Aldehyde oxidase derived from rabbit liver has a wide substrate range and produces a much smaller proportion of 4-pyridone on oxidation of N\textsubscript{1}-methylnicotinamide than the corresponding enzyme from other mammalian sources.\textsuperscript{9,10,39} In this investigation both rabbit and guinea pig will be used as sources of aldehyde oxidase. The research interests of this current investigation can be divided into three main areas.
(i) **Product identification**

Previous workers\(^3\) had used ultraviolet spectroscopy to identify N-methyl-2-quinolone as the only oxidation product of N-methylquinolinium with rabbit liver aldehyde oxidase. In view of the findings with N\(^1\)-methylnicotinamide the initial aim was to reinvestigate the oxidation of N-methylquinolinium to determine if any N-methyl-4-quinolone is also produced. Also of particular interest would be the oxidation product(s) of N-phenylquinolinium (i.e. where a bulky group replaces methyl) should it be found to be a substrate. Any products produced from other substituted quinolinium and related cations will also require identification. It is intended to develop a sensitive high pressure liquid chromatographic method for the separation and isolation of oxidation products formed. In addition to chromatographic methods, further confirmation of product identity would be afforded using a combination of infrared, nuclear magnetic resonance, ultraviolet and mass spectral techniques. In most cases methods have already been developed to obtain clearly resolved spectra from less than milligram quantities of material.\(^{144}\) It will be necessary to synthesise the N-heterocyclic salts and authentic samples of the anticipated enzymic oxidation products.

(ii) **Kinetics**

The rates of oxidation and Michaelis–Menten constants for the N-heterocyclic cations under consideration will all be determined and compared with data previously recorded for the parent unquaternised compounds.\(^6\) It is also intended to study the oxidation of a N-heterocyclic cation and a non-quaternary compound over a wide pH range. It has been previously reported that for aldehyde oxidase as pH increases \(K_m\) values for charged substrates decrease, whereas \(K_m\) values for uncharged substrates increase, but no detailed account of \(K_m\) values or pH ranges were given.\(^3\) From monitoring the oxidation of a N-heterocyclic cation in the presence
of an uncharged substrate, it is hoped that it will be possible to determine whether these two types of substrate have a common binding site on the enzyme. To achieve these objectives will involve further development of spectrophotometric techniques previously used to obtain kinetic data.29

(iii) Pathway of oxidation

Pseudobase formation plays an important role in the chemistry of N-heterocyclic cations.145 Pseudobases are often intermediates in oxidation reactions.146

![Diagram of pseudobase formation]

The pH at which pseudobase formation occurs is related to the structure of the N-heterocyclic cation.147 Felsted et al. postulated that pseudobases of N1-methylnicotinamide were intermediates in the aldehyde oxidase catalysed oxidation of this cation.44 This investigation aims to determine whether pseudobases can act as substrates for aldehyde oxidase. This will require synthesis of a pseudobase which does not equilibrate to give significant amounts of the corresponding N-heterocyclic cation and is stable at pH 7.
Some of the N-heterocyclic cations to be studied are shown below:

- N-Methylquinolinium
- N-Phenylquinolinium
- N-Methyl-5,6-benzoquinolinium
- N-Methyl-7,8-benzoquinolinium
- N-Methyl-phenanthridinium
- N-Methyl-4-methylquinolinium
- N-Methyl-2-methylquinolinium
- N-Methyl-4-phenylquinolinium
- N-Methyl-2-phenylquinolinium
- N-Phenyl-2-phenylquinolinium
CHAPTER 2

Experimental

BIOCHEMICAL PROCEDURES

2.1 Materials

G75 Sephadex
CX 10 Ultrafiltration Units
Protein Assay Dye Reagent
Silica Gel
Membrane Filters

Pharmacia Fine Chemicals, Hounslow, U.K.
Millipore, London, U.K.
Biorad, Watford, U.K.
Merck, B.D.H., Liverpool, U.K.
Sartorius, Belmont Sutton, U.K.

Xanthine Oxidase, Grade 1 from Buttermilk
Catalase, Purified Powder from Bovine Liver
Cytochrome c, Type III from Horse Heart
DEAE Cellulose

Sigma Chemical Company Ltd.
Gillingham, U.K.

2.2 Enzyme Preparation

2.2.1 Partially purified aldehyde oxidase

Aldehyde oxidase was prepared from two sources, New Zealand White female rabbits and Dunkin Hartley female guinea pigs. The animals were killed by fracturing the neck; the liver was removed immediately and the gall bladder rejected. The weighed liver was homogenized for two minutes in three times its volume of an ice-cooled 1.15% solution of potassium chloride containing $10^{-4}$ M EDTA. The resultant suspension was transferred to two 500 ml conical flasks and heated on a water bath at 55-60°C for 10 min. After cooling in an ice-bath to ca. 10°C the suspension was centrifuged 15,000 g for 45 min at 4°C using a MSE model 18 centrifuge. The supernatant was filtered through glass wool into a measuring cylinder to give a clear red solution. The volume of supernatant was noted and the
enzyme precipitated by the addition of solid ammonium sulphate to 50% saturation (i.e. 35.3 g per 100 ml). The solution was stirred for 30 min on an ice-bath until all the ammonium sulphate had dissolved and then was centrifuged at 10,000 g for 20 min at 4°C. The precipitate so collected was dissolved in the minimum amount of $10^{-4}$ M EDTA solution (5-10 ml). The enzyme was kept at ca. 0°C (on ice/water) and used immediately or stored in liquid nitrogen where it retained its activity for several months. This partially purified preparation of aldehyde oxidase was used in product identification and kinetic studies.

2.2.2 Further purification

The ammonium sulphate fraction from rabbit liver was further purified at 0-5°C as follows:

- $n_m$ ml of the ammonium sulphate fraction was applied on to a G75 Sephadex column (50 cm x 1.5 cm i.d.) and eluted with a $10^{-4}$ M EDTA solution. Active fractions (see section 2.6.1) were combined and concentrated using CX 10 ultrafiltration units and applied to a DEAE cellulose column (50 cm x 1.5 cm i.d.). The column was eluted with 100 ml of 0.1 M potassium phosphate buffer pH 7.8 containing $10^{-4}$ M EDTA, followed by 0.2 M potassium phosphate buffer pH 7.8, also containing $10^{-4}$ M EDTA. Aldehyde oxidase activity was eluted with the 0.2 M buffer and the fractions were again concentrated by ultrafiltration.

Aldehyde oxidase is known to be susceptible to poisoning by heavy metals. Consequently all solutions used in experiments with the enzyme contained $10^{-4}$ M EDTA (ethylenediaminetetraacetic acid, disodium salt), a metal chelating agent.
2.3 **Protein Determination**

The protein concentration for each enzyme preparation was routinely determined using the method of Bradford,\(^{148}\) which is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Calibration curves were obtained using bovine serum albumin.

2.4 **Incubation and Extraction Procedures**

2.4.1 **For product identification**

(i) Unless otherwise stated, the substrate (5-15 \(\mu\)mol) was incubated with partially purified aldehyde oxidase (equivalent to 1 g of liver) made up to a total volume of 11.25 ml with Sørensen's phosphate buffer, pH 7, 0.067 M. The incubations were carried out in air, for 1 h at 37°C in a shaking water bath. Incubations with xanthine oxidase (0.1 ml; Grade 1, 16.6 units/ml, 28 mg protein/ml) were carried out in a similar fashion.

Control incubations were carried out as follows with (a) substrate or anticipated metabolites and denatured enzyme (heated to 100°C for 10 min), (b) mixtures containing all the components except the substrate in the same way as described for the test mixtures. In addition, anticipated metabolites were also incubated with active enzyme to determine whether these were the final products of aldehyde oxidase catalysed oxidation or whether they could be further oxidised.

The reactions were terminated by the addition of sufficient sodium sulphate to precipitate the protein, followed by heating on a boiling water bath for 20 min. The resulting mixtures were extracted with dichloromethane (4 x 25 ml) and in each case the combined extracts evaporated down to a final volume of 1 ml. Aliquots of these dichloromethane solutions were then subjected to thin layer and high pressure liquid chromatography.
(ii) In order to obtain sufficient quantity of product for nuclear magnetic resonance (n.m.r.) spectra, large scale incubations were carried out. The substrate (0.1-0.2 mmol) was incubated with partially purified aldehyde oxidase (equivalent to 2 g of liver) made up to a total of 25 ml with Sørensen's phosphate buffer, pH 7 0.067 M. The incubations were performed in air for 24 h at 37°C in a shaking water bath and both controls and extractions were carried out as in (i). The product(s) was then isolated from the concentrated dichloromethane extract using preparative chromatographic plates (section 2.5.1).

2.4.2 For product ratio determinations

Determinations of product ratios under a variety of conditions (described in sections 3.4 and 4.5) were carried out using N-methylquinolinium perchlorate (usually 1.25 μmol or 12.5 μmol) and rabbit liver aldehyde oxidase (equivalent to 2 g of liver) in a total volume of 5 ml. Unless otherwise stated incubations were performed at pH 7 in Sørensen's phosphate buffer, 0.067 M.

The solutions were incubated in air for 1 h in a shaking water bath (except for incubations employing potassium ferricyanide (10^-2 M) as the electron acceptor, which were carried out anaerobically under nitrogen). Each reaction was then terminated by the addition of 5 ml of methanol which precipitates the protein and is also a known inhibitor of aldehyde oxidase. The resulting mixtures were then centrifuged at 3,000 g for 5 min in a Beckman Microfuge B centrifuge to remove suspended coagulated protein material. Aliquots of aqueous methanolic supernatant were injected directly on to a reverse phase high pressure liquid chromatography (h.p.l.c.) column (system p5, see Table 2.1).

Control experiments involved incubating (i) substrate or anticipated products under the particular condition to be tested but with denatured
enzyme (heated to 100°C for 10 min), (ii) mixtures containing all the components except the substrate in the same way as described for the test mixtures.

In order to obtain a progress curve for the production of N-methyl-2-quinolone and N-methyl-4-quinolone the following experiment was carried out.

A solution was prepared of partially purified aldehyde oxidase (equivalent to 2 g of liver) and catalase (10 mg) in 50 ml of Sørensen's phosphate buffer, pH 7, 0.067 M (catalase was used to destroy any hydrogen peroxide formed during the course of the determination, which might cause inactivation of the enzyme). A solution of N-methylquinolinium perchlorate (32 mg) in 50 ml of pH 7 buffer was also prepared. After equilibrating the two solutions separately for 5 mins at 30°C, they were then mixed together to start the reaction (t = 0) and then maintained at 30°C in a shaking water bath for one hour. Portions (0.25 ml) were withdrawn from the reaction mixture at fixed time intervals which were added to 0.25 ml of ice cold methanol. The resultant suspension was centrifuged at 3,000 g for 30 s and then stored on ice until used. Aliquots of the supernatant were injected directly on to the h.p.l.c. column, system p5.

2.5 Identification of Incubation Products

Unless otherwise stated, incubation mixtures or their dichloromethane extracts, were examined by the following techniques.

2.5.1 Thin layer chromatography (t.l.c.)

Glass plates coated with silica gel G254 (0.25 mm thickness) which contained a fluorescent indicator for irradiation at 254 nm were used for thin layer chromatography. Aliquots of the incubation extracts (as obtained in section 2.4.1(i)), together with the relevant authentic
reference compounds, were applied to the plates which were developed to 10 cm in a number of different solvent systems at room temperature. The solvent systems employed in this study are given below:

<table>
<thead>
<tr>
<th>Solvent Mixture</th>
<th>(v:v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Butan-1-ol/gl. acetic acid/water</td>
<td>60:20:20</td>
</tr>
<tr>
<td>B Methanol/chloroform</td>
<td>5:95</td>
</tr>
<tr>
<td>C Isoamyl acetate</td>
<td></td>
</tr>
<tr>
<td>D Methanol/chloroform</td>
<td>10:90</td>
</tr>
<tr>
<td>E Propan-1-ol/cyclohexane</td>
<td>20:80</td>
</tr>
<tr>
<td>F Methanol/dichloromethane</td>
<td>5:95</td>
</tr>
<tr>
<td>G Propan-1-ol/gl. acetic acid/water</td>
<td>60:25:15</td>
</tr>
<tr>
<td>H Propan-2-ol/cyclohexane</td>
<td>20:80</td>
</tr>
<tr>
<td>I Propan-2-ol/cyclohexane</td>
<td>30:70</td>
</tr>
</tbody>
</table>

Chromatograms were examined under an ultraviolet lamp at 253.7 nm and 375 nm. Spots were detected by quenching of the fluorescent background at 253.7 nm, indicated by "+" in the tables reporting t.l.c. data in Chapter 3, and by native fluorescence at 375 nm (colour of which is reported). In these tables the symbol "s" indicates streaking rather than a discrete spot.

Products resulting from large scale incubations (see section 2.4.1(i)) were isolated using silica gel G<sub>254</sub> plates (1 mm thickness). The spots corresponding to product were scraped off and eluted from the silica with dichloromethane. The eluates were dried over MgSO<sub>4</sub> and then evaporated to dryness. The residue thus obtained was subjected to analysis by n.m.r.

2.5.2 **High pressure liquid chromatography (h.p.l.c.)**

High pressure liquid chromatographic analysis was carried out using
a Dupont 810 pump equipped with a variable wavelength u.v. detector and digital Venture Mark II integrator. The apparatus was fitted with a fixed volume Rheodyne loop (20 μl).

A number of adsorption and reverse phase systems were developed, details of which are given in Table 2.1 and used to:

(i) To separate incubation products and compare their retention volumes with those for authentic reference compounds. If the compound was not found to be eluted from the column this is denoted "n" in the h.p.l.c. data tables in Chapter 3.

(ii) To quantify the amount of product formed. This was achieved by comparing the integrated peak areas of the incubation products against calibration curves of integrated peak area versus concentration for the authentic reference compounds.

(iii) Isolate product. The eluate corresponding to the peak obtained for the incubation product was collected in a 5 ml reacti-vial and the solvent evaporated by heating on a hot plate. The process was repeated until a sufficient amount of product for infrared and mass spectral studies had been collected.

2.5.3 Infrared spectra

Infrared spectra were recorded on a Perkin Elmer 297 instrument fitted with a beam condenser, microdisc holder and beam attentuator.

Three to 4 mg of previously ground potassium chloride was weighed on to a small piece of aluminium foil and placed underneath an infrared lamp. The compound collected from h.p.l.c. was dissolved in 20 to 30 μl of dichloromethane and the solution added dropwise to the potassium chloride. When all the solvent had evaporated the potassium chloride was placed carefully into the microdisc die. The die was evacuated for one minute and enough pressure applied to form a transparent disc. The disc was then transferred to the microdisc holder.
<table>
<thead>
<tr>
<th>Phase</th>
<th>Column*</th>
<th>Eluting Solvent†</th>
<th>(v:v)</th>
<th>System</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad</td>
<td>Shandon Hypersil 5 µ</td>
<td>ethanol/cyclohexane</td>
<td>7:3</td>
<td>a₁</td>
</tr>
<tr>
<td>Ad</td>
<td>&quot;</td>
<td>ethanol/cyclohexane</td>
<td>1:1</td>
<td>a₂</td>
</tr>
<tr>
<td>Ad</td>
<td>&quot;</td>
<td>propan-2-ol/cyclohexane</td>
<td>3:7</td>
<td>a₃</td>
</tr>
<tr>
<td>Ad</td>
<td>Dupont Zorbax 5 µ</td>
<td>propan-2-ol/cyclohexane</td>
<td>1:4</td>
<td>a₄</td>
</tr>
<tr>
<td>Ad</td>
<td>&quot;</td>
<td>methanol/dichloromethane</td>
<td>1:9</td>
<td>a₅</td>
</tr>
<tr>
<td>Ad</td>
<td>Dupont Zorbax CN</td>
<td>ethanol/cyclohexane</td>
<td>1:1</td>
<td>a₆</td>
</tr>
<tr>
<td>Ad</td>
<td>&quot;</td>
<td>ethanol/cyclohexane</td>
<td>1:9</td>
<td>a₇</td>
</tr>
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<td>&quot;</td>
<td>ethanol/cyclohexane</td>
<td>1:19</td>
<td>a₈</td>
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<tr>
<td>Ad</td>
<td>&quot;</td>
<td>propan-2-ol/cyclohexane</td>
<td>1:9</td>
<td>a₉</td>
</tr>
<tr>
<td>Ad</td>
<td>&quot;</td>
<td>acetonitrile/dichloromethane</td>
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<td>a₁₀</td>
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<tr>
<td>Pt</td>
<td>&quot;</td>
<td>acetonitrile/water</td>
<td>1:1</td>
<td>p₁</td>
</tr>
<tr>
<td>Pt</td>
<td>&quot;</td>
<td>acetonitrile/water</td>
<td>1:49</td>
<td>p₂</td>
</tr>
<tr>
<td>Pt</td>
<td>&quot;</td>
<td>acetonitrile/water/gl. acetic acid</td>
<td>5:45:0:05</td>
<td>p₃</td>
</tr>
<tr>
<td>Pt</td>
<td>Shandon Hypersil ODS 5 µ†</td>
<td>methanol/Sørensen's phosphate buffer pH 7</td>
<td>1:1</td>
<td>p₅</td>
</tr>
</tbody>
</table>

* Column length in each case 25 cm x 4.6 mm i.d. Flow rate 2 ml min⁻¹. Monitored at 325 nm unless otherwise stated. Ad - Adsorption phase. Pt - Partition/reverse phase.
† Prior to use solvents (h.p.l.c. grade when available) were filtered through membrane micropore filters and then degassed.
† Connected via a pre-column (5 cm x 4.6 mm i.d.) of the same packing.
2.5.4 **Mass spectra**

Mass spectra were measured on an AEI MS 902 Mass Spectrometer by Mr. Roger Nettleton at Bradford University.

The samples isolated from h.p.l.c. were subjected to mass spectral analysis; this involved dissolving the residues in 10-20 µl of dichloromethane and evaporating the solution directly on to the probe tip.

2.5.5 **Ultraviolet spectra**

Ultraviolet (u.v.) spectra were recorded using the spectrophotometer described in section 2.6. Spectra of incubation products were either determined directly on eluates from partition phase h.p.l.c. systems or after evaporating eluates from adsorption phase systems to dryness and redissolving the residue in pH 7 phosphate buffer. Spectra of these solutions were also recorded after adjustment to pH 1 with hydrochloric acid.

2.5.6 **Nuclear magnetic resonance spectra**

Nuclear magnetic resonance (n.m.r.) spectra were recorded by Dr. Paul Pringle at Leeds University on a JEOL FX100 NMR spectrometer with deuterium used to provide field/frequency lock. Spectra were measured at +21°C 100 MHz and referenced to δ(Me₄Si) = 0.000 p.p.m.

2.6 **Spectrophotometric Assays**

All kinetic work, protein determinations and ultraviolet spectra were recorded using a Pye Unicam Sp 8 250 spectrophotometer fitted with a thermostatted cell holder which was kept at 30°C.

Unless otherwise stated, all the solutions used in the kinetic studies were made up in Sörensen's phosphate buffer pH 7, 0.067 M and kept in a water bath at 30°C to minimise temperature effects on mixing. Enzyme preparations were kept at ca. 0°C (ice/water) prior to addition to the cuvette.
2.6.1 **Enzyme activity**

The activity of the enzyme fractions arising from the purification procedure (section 2.2.2) were tested by monitoring the increase in absorbance at 322 nm due to the production of 9-phenanthridone from phenthridine. 0.1 ml of the fraction to be tested was added to the cuvette which contained 5 x 10^{-5} M phenanthridine and to the reference cuvette which contained only buffer (total volume in each cuvette was 3 ml).

2.6.2 **Measurement of substrate oxidation rates**

Partially purified preparations of aldehyde oxidase were used throughout these studies.

2.6.2.1 **Potassium ferricyanide as electron acceptor**

The rate of oxidation of the substrate was monitored by following the decrease in absorbance at 420 nm resulting from reduction of ferricyanide to ferrocyanide. The reaction cuvette contained:

- *Potassium ferricyanide* - 1 x 10^{-3} M
- *Substrate* - variable concentration
- *Enzyme* - sufficient to produce a suitable rate of reaction and hence a change in absorbance during time of observation
- *Sørensen's phosphate buffer, pH 7, 0.067 M* to a total volume of 3 ml

The reference cuvette contained all components except the substrate. The rate of substrate oxidation with xanthine oxidase was determined in a similar fashion.

All substrates were tested for non-enzymatic reduction of ferricyanide under the above conditions, the enzyme being omitted from the cuvettes. An additional control was performed omitting potassium ferri-
cyanide to ensure enzymatic oxidation of the substrate did not result in any change in absorbance at 420 nm in the absence of this reagent.

Rates measured in the presence of inhibitor were carried out as above except that inhibitor was also added to both test and reference cuvettes.

2.6.2.2 Cytochrome c as electron acceptor

Reaction conditions were the same as those in section 2.6.2.1 except that cytochrome c $2 \times 10^{-5}$ M replaced potassium ferricyanide and reduction of cytochrome c was monitored by the corresponding increase in absorbance at 550 nm.

2.6.2.3 Oxygen as electron acceptor

In certain cases the ultraviolet absorption spectra of a substrate and its corresponding oxidation product were suitably different to be able to monitor the change in absorbance at a single wavelength due to either depletion of substrate or formation of product. The wavelength monitored for a particular substrate is recorded in the relevant section.

Reaction conditions were the same as those in section 2.6.2.1 except that potassium ferricyanide was omitted.

2.6.2.4 Effect of pH on rate of reaction

Rate determinations were carried out for the substrate with partially purified aldehyde oxidase within the pH range 5.4-10.2. For each pH profile the same batch of enzyme was used throughout. Unless otherwise stated, the electron acceptor employed was ferricyanide and the reaction conditions were as described in section 2.6.2.1 with the following changes:

(i) Enzyme was diluted in $10^{-4}$ M EDTA solution rather than buffer solution.
(ii) Assays in the pH range 5.4-8.2 were conducted in Sørensen's phosphate buffer 0.067 M. Assays in the pH range 8.4-10.2 were conducted in Sørensen's glycine buffer II.

2.6.3 Specific activity

Specific activity is expressed as the number of μmoles of substrate transformed per min per mg of protein at 30°C. This is calculated from the $V_{\text{max}}$ value obtained for the substrate with enzyme of known protein concentration (unless otherwise stated the $V_{\text{max}}$ value was determined from the Lineweaver-Burk plot of the kinetic data).

(i) Using the ferricyanide assay

The molar extinction change at 420 nm for reduction of ferricyanide to ferrocyanide is 1040, and does not vary over the pH range 5-10. However, for each mole of substrate hydroxylated 2 moles of ferricyanide are reduced. The extinction change per mole of substrate hydroxylated is therefore 2080. The number of μmoles of substrate oxidised per min was then calculated from the absorbance change observed per min using the relationship:

$$c = \frac{A}{\epsilon_l}$$

where $c$ = concentration (M), $A$ = absorbance, $l$ = path length of absorbing solution in cm (1 cm silica cuvettes were used), $\epsilon$ = molar extinction coefficient, which has units of 1000 cm$^2$ mol$^{-1}$ but the units are, by convention, never expressed.

(ii) Using the cytochrome c assay

Massey$^{149}$ reported the molar extinction change at 550 nm for the reduced form of horse heart cytochrome c to be 21,000 ($\epsilon_{550}$ reduced-oxidised). However, for each mole of substrate hydroxylated 2 moles of cytochrome c are reduced. The extinction change per mole of substrate hydroxylated is therefore 42,000.
CHEMICAL SYNTHSES

2.7 Materials

All chemicals required for the syntheses below were purchased from Aldrich Chemical Company Limited, Gillingham, Dorset, U.K.

2.8 Preparation of N-Heterocyclic Salts*

2.8.1 N-Methyl quaternary compounds

Preparation of N-methylquinolinium, N-methyl-2-methylquinolinium, N-methyl-4-methylquinolinium, N-methyl-5,6-benzoquinolinium, N-methyl-7,8-benzoquinolinium, N-methylisoquinolinium and N-methylphenanthridinium salts

(i) Iodide salts of the N-heterocyclic cations listed were prepared by refluxing the appropriate heterocyclic base with excess iodomethane in ethanol for ca. 2 h (except in the case of N-methyl-7,8-benzoquinolinium iodide which was prepared similarly but in a closed vessel). The solvent was then removed under reduced pressure; the resulting solid after washing with acetone and ether was recrystallised from ethanol to give high yields (85-95%) of the required product.

(ii) The N-heterocyclic chlorides listed in Table 2.2 were obtained from the corresponding iodide salt by one of two methods, depending on the solubility of the latter salt in water; if soluble then method (a) was used:

(a) The N-heterocyclic iodide was dissolved in water and passed down a Dowex 1-X8 ion-exchange column (chloride form) the eluate being monitored by a u.v. detector. The fraction containing the desired compound was evaporated to dryness and the solid so obtained was recrystallised from ethanol to give the chloride salt in high yield.

* Melting points reported in this section and in section 2.9 are uncorrected.
Table 2.2. Melting points for N-methyl heterocyclic salts.

<table>
<thead>
<tr>
<th>Cation</th>
<th>Iodide</th>
<th>Chloride m.p.(^a)/°C</th>
<th>Perchlorate</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-methylquinolinium</td>
<td>133(133(^151))</td>
<td>125(126(^151))</td>
<td>117-118(117.5-118.5(^152); 122(^153))</td>
</tr>
<tr>
<td>N-methyl-2-methylquinolinium</td>
<td>195(195(^151))</td>
<td>n.p.</td>
<td>153-154(152-154(^154))</td>
</tr>
<tr>
<td>N-methyl-4-methylquinolinium</td>
<td>174(172-174(^155))</td>
<td>n.p.</td>
<td>161(162-163(^155))</td>
</tr>
<tr>
<td>N-methyl-5,6-benzoquinolinium</td>
<td>201-204(200-205(^151))</td>
<td>234-236(236(^151))</td>
<td>226(^b)</td>
</tr>
<tr>
<td>N-methyl-7,8-benzoquinolinium</td>
<td>176-177(179(^151))</td>
<td>134(133(^151))</td>
<td>136-138(^b)</td>
</tr>
<tr>
<td>N-methylisoquinolinium</td>
<td>159-160(159(^151))</td>
<td>148-150(155(^157))</td>
<td>113-115(117(^153))</td>
</tr>
<tr>
<td>N-methylphenanthridinium</td>
<td>202(204.5(^151))</td>
<td>213(215(^150))</td>
<td>194-195(192-195(^156))</td>
</tr>
</tbody>
</table>

\(^a\) Literature reported melting point and reference in parentheses.

\(^b\) Elemental analysis for these salts which have not been previously prepared: N-methyl-5,6-benzoquinolinium perchlorate
(C\(_{14}H_{12}ClNO_4\) requires: C, 57.19; H, 4.08; Cl, 12.35; N, 4.79. Found: C, 57.24; H, 4.09; Cl, 12.09; N, 4.77)
N-methyl-7,8-benzoquinolinium perchlorate (C\(_{14}H_{12}ClNO_4\) requires: C, 57.19; H, 4.08; Cl, 12.35; N, 4.79. Found: C, 56.84; H, 4.12; Cl, 11.90; N, 4.80)

n.p. - not prepared
(b) The N-heterocyclic iodide (1 g) and 1.5 molar equivalents of freshly prepared silver chloride were stirred and refluxed in water (80 ml) for 3 h in subdued light. After filtering the solution the filtrate was evaporated to dryness under reduced pressure to give a solid. N-Methylphenanthridinium and N-methyl-7,8-benzoquinolinium chlorides were recrystallised from aqueous ethanol. N-Methyl-5,6-benzoquinolinium chloride was recrystallised from ethanol/ether. Yields were between 50 and 60%.

(iii) Perchlorate salts were prepared by dissolving the appropriate N-heterocyclic iodide in the minimum of hot water and adding dropwise a solution of sodium perchlorate (a 5-fold molar excess) dissolved in the minimum of cold water. On cooling, the perchlorate salt which crystallised was filtered off and recrystallised from water to give excellent yields (90-100%) of the required product.

The melting points of the N-heterocyclic salts are given in Table 2.2 and compared with those reported in the literature. The n.m.r. data are given in Table 2.3.

Table 2.3. N.m.r. data for some N-methyl heterocyclic compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>( \delta(N-CH_3) )</th>
<th>( \delta(\text{Aromatics}) )</th>
<th>( \delta(\text{others}) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-methylquinolinium+ X^-</td>
<td>4.81</td>
<td>8.11-9.01</td>
<td></td>
</tr>
<tr>
<td>N-methyl-2-methylquinolinium+ X^-</td>
<td>4.61</td>
<td>7.71-8.80</td>
<td>3.21^c</td>
</tr>
<tr>
<td>N-methyl-4-methylquinolinium+ X^-</td>
<td>4.78</td>
<td>7.91-9.76</td>
<td>3.02^d</td>
</tr>
<tr>
<td>N-methyl-5,6-benzoquinolinium+ X^-</td>
<td>4.52</td>
<td>7.67-9.68</td>
<td></td>
</tr>
<tr>
<td>N-methyl-7,8-benzoquinolinium+ X^-</td>
<td>5.19</td>
<td>7.70-9.55</td>
<td></td>
</tr>
<tr>
<td>N-methylisoquinolinium+ X^-</td>
<td>4.66</td>
<td>7.68-8.61</td>
<td></td>
</tr>
<tr>
<td>N-methylphenanthridinium+ X^-</td>
<td>4.86</td>
<td>7.66-8.84</td>
<td></td>
</tr>
</tbody>
</table>

^a Measured in CDCl_3, \( \delta \) values in p.p.m.  
^b \( X = \text{ClO}_4 \)  
^c \( \delta(C_2-CH_3) \)  
^d \( \delta(C_4-CH_3) \)
Preparation of N-methyl-4-phenylquinolinium salts

4-Phenylquinoline picrate.\textsuperscript{158} β-Chloropropiophenone (16.8 g, 0.1 mol), aniline (18.6 g, 0.2 mol), aniline hydrochloride (12.95 g, 0.1 mol) and ethanol (13 ml) were heated and stirred together at 100°C for 1 h. The mixture was then made alkaline with potassium hydroxide. The base was extracted with ether, dried over MgSO\(_4\) and the ether evaporated off to yield a yellow oil which was then vacuum distilled. The fraction (10 g) b.p. 100-290°C/20 mm was heated for 1 h at 100°C with acetic anhydride (12 g) and the resulting mixture extracted with dichloromethane. The combined extracts were filtered; the filtrate was dried over MgSO\(_4\) and then evaporated to yield an oil, which was treated with ethanolic picric acid solution. 4-Phenylquinoline picrate separated from ethanol as yellow needles (yield 4 g, 10%)

m.p. 222°C  Literature reports 225°C,\textsuperscript{158} 224°C,\textsuperscript{159} 223-226°C\textsuperscript{160}

4-Phenylquinoline.\textsuperscript{160,161} 4-Phenylquinoline picrate (3 g, 6.9 mmol) was added to 100 ml of a saturated solution of lithium hydroxide (ca. 13%). This solution was stirred and warmed; after cooling, the solution was extracted with ether. The combined extracts were washed twice with a saturated solution of lithium hydroxide, dried over MgSO\(_4\) and then evaporated to yield 4-phenylquinoline as an oil (1.2 g)

Mass spectrum: m/e 205(M)

N-Methyl-4-phenylquinolinium iodide was prepared from 4-phenylquinoline (0.5 g, 2.4 mmol) and iodomethane (1 g, 7 mmol) in ethanol (10 ml) by the method described on page 60, section (i). The product was recrystallised from water to yield orange needles which were dried in vacuo (yield 0.5 g, 59%)

m.p. 219-220°C  Literature reports 220.5-221.5°C,\textsuperscript{160} 222°C\textsuperscript{159}

N-Methyl-4-phenylquinolinium perchlorate was prepared from the iodide salt by the method outlined on page 62, section (iii). The product
was recrystallised from water to yield white crystals which were dried in vacuo

m.p. 184-185°C

No previous reports of the preparation of this compound were found in the literature. Confirmation of the identity of the product was afforded by elemental analysis.

Analysis:  
C, 60.02; H, 4.42; N, 4.44

\( \text{C}_{16}\text{H}_{14}\text{NO}_4\text{Cl} \) requires:  
C, 60.00; H, 4.38; N, 4.38

**Preparation of N-methyl-2-phenylquinolinium salts**

*N-Methyl-2-phenylquinolinium iodide*. A clear solution of phenylmagnesium bromide [prepared from bromobenzene (8 g, 0.05 mol) and magnesium (1.2 g, 0.05 mol)] was slowly transferred under nitrogen pressure using a needle and tubing into a stirred solution of N-methylquinolinium iodide (13 g, 0.05 mol) in ether (100 ml). An exothermic reaction ensued which required cooling. A solution of iodine (12.5 g, 0.1 mol) and sodium acetate (10 g, 0.12 mol) in warm alcohol (ca. 200 ml) was then added to the mixture and immediately a yellow solid precipitated, which was filtered and washed with water. The product was recrystallised twice from ethanol to yield orange needles (yield 14 g, 85%)

m.p. 197-198°C  
Literature reports 200°C, 163 197°C164

*N-Methyl-2-phenylquinolinium perchlorate* was prepared from the iodide salt by the method outlined on page 62, section (iii). The product was recrystallised from water and the white crystalline solid dried in vacuo.

m.p. 218-219°C

N.m.r. (CDCl\(_3\)) \( \delta \) 4.59 (s, N-CH\(_3\)), 7.71-8.97 (m, aromatic-H)

Elemental analysis  
C, 60.00; H, 4.38; N, 4.38; Cl, 11.11

\( \text{C}_{16}\text{H}_{14}\text{NCIO}_4 \) requires:  
C, 60.31; H, 4.33; N, 4.34; Cl, 11.08
Preparation of 3-methyl-2-oxoquinazolinium salts

3-Methyl-2-oxoquinazolinium iodide. 2-Hydroxyquinazoline (0.55 g, 3.8 mmol), iodomethane (2 ml) and methanol (5 ml) were heated in a sealed tube at 100°C for 3 h. After cooling, the orange crystals were filtered and washed with acetone and ether (yield 0.14 g, 13%) m.p. 238-240°C Literature reports 238-239.5°C

Infrared spectrum (KBr) ν(CO) 1740 cm⁻¹, ν(CN) 1640 cm⁻¹

o-Nitrobenzaldehyde dimethylacetal. A mixture of o-nitrobenzaldehyde (38 g, 0.25 mol), dimethyl sulphite (30 g, 0.27 mol) and methanol (60 ml) was treated with a trace of dry hydrogen chloride and refluxed on a water bath for 8 h, sulphur dioxide being evolved as acetalisation took place. The homogeneous mixture was poured into a solution of sodium methoxide (sodium 1 g; methanol 20 ml) to neutralise free acid present, and the excess dimethyl sulphite was decomposed by shaking for 30 min with 15% sodium hydroxide solution (100 ml). The mixture was diluted to 500 ml with water and the acetal extracted with ether and washed with water. The ether was evaporated to yield a liquid (44.7 g; 91%).

o-Aminobenzaldehyde dimethylacetal. The o-nitrobenzaldehyde dimethylacetal (44 g, 0.22 mol) was slowly poured down a condenser into a hot agitated mixture prepared from crystalline sodium sulphide (150 g in 150 ml of water) and concentrated hydrochloric acid (62.5 g). Reaction commenced almost immediately and was completed by boiling for a further 8 h. The required product was extracted with ether, washed with water and the combined ether extracts evaporated to yield a liquid (24 g, 64%). This product was used immediately in the next step since it readily decomposes to the free aldehyde.

o-Aminobenzaldehyde dimethyl acetal-N-methyl urea. Methyl isocyanate (8.15 g, 0.143 mol) was added dropwise to a stirred solution of o-aminobenzaldehyde dimethylacetal (24 g, 0.143 mol) in ether (50 ml).
Triethylamine ($\alpha, 0.5 \text{ ml}$) was added to the solution which was stirred at room temperature for 14 h. The white crystals that had precipitated were filtered and recrystallised from toluene (yield 27.3 g, 85%).

m.p. 120-122°C Literature reports$^{167}$ 124-126°C

3-Methyl-2-oxoquinazolinium chloride. Concentrated hydrochloric acid was dripped into a stirred solution of the above urea (11.2 g, 0.05 mol) in dioxan (50 ml) at 70-80°C, producing a yellow precipitate. Further acid was added until the pH of the mixture was $\alpha, 2$. The solid was dissolved in water, and filtered; the filtrate was evaporated and the solid product dried over $\text{P}_2\text{O}_5$ (yield 4.5 g, 46%).

m.p. 248°C Literature reports 245°C$^{167}$

Infrared (nujol) $\nu$(CO) 1740 cm$^{-1}$, $\nu$(CN) 1640 cm$^{-1}$

N.m.r. (CDCl$_3$) $\delta$ 4.1 (s, CH$_3$), 7.5-7.8 (m, aromatic-H), 8.0-8.4 (m, aromatic-H), 9.9 (s, C$_1$-H)

3,4-Dihydro-4-hydroxy-3-methyl-2-quinazolinone. 3-Methyl-2-oxoquinazolinium chloride (1 g, 5 mmol) dissolved in water (10 ml) was neutralised by adding aqueous sodium hydroxide (1 M). A white solid was precipitated which was filtered, washed with water and dried over $\text{P}_2\text{O}_5$ (yield 0.68 g, 75%).

m.p. 185-188°C Literature reports 189-190°C, $^{167}$ 192-194°C$^{168}$

Infrared spectrum (nujol) $\nu$(NH, OH) 3300-3000 cm$^{-1}$, $\nu$(CO) 1660 cm$^{-1}$

N.m.r. (DMSO $d_6$) $\delta$ 2.9 (s, CH$_3$), 5.5-5.7 (d, 0-H), 6.1-6.3 (d, N$_3$CH), 6.6-7.3 (m, aromatic-H), 9.5 (s, broad, N$_1$-H)

Preparation of 3,4-dihydro-4-hydroxy-3-methylquinazoline from 3-methyl-quinazolinium iodide$^{169,170}$

Quinazoline (1.5 g) and iodomethane (1.5 ml) were added together and left to stand overnight. Excess iodomethane was removed on a steam bath; the resultant solid was dissolved in water (15 ml) and treated, with cooling, with 33% aqueous potassium hydroxide (3 ml). The pseudobase that
crystallised in needles was filtered off, washed with water until the filtrate was no longer alkaline and dried at 100°C to constant weight (yield 1 g, 36%)

- m.p. 162°C  
- Literature reports 161-162°C, 164-165°C, 167-168°C

- Mass spectrum: m/e 161 (M) 144 (M-OH)

2.8.2 N-Phenyl quaternary compounds

Preparation of N-phenylquinolinium perchlorate

Diphenylamine (34 g, 0.2 mol), nitrobenzene (50 ml), dry toluene (70 ml) and concentrated hydrochloric acid (24 ml) were stirred under nitrogen, producing a thick yellow salt suspension. Acrolein (13 g, 0.22 mol) in 30 ml of a 50/50 nitrobenzene/toluene solution was added to the mixture over 3 h and the resulting red salt solution stirred for a further 1 h. The toluene and nitrobenzene were removed by steam distillation and the remaining aqueous phase boiled over charcoal and then hot filtered. Addition of perchloric acid to the filtrate gave a precipitate of N-phenylquinolinium perchlorate which was filtered off, recrystallised from water and finally dried in vacuo (yield 23 g, 37%)

- m.p. 157°C  
- Literature reports 157°C, 155-156°C

- N.m.r. (CDCl₃) 57.66-9.30, (m, aromatic-H)

Preparation of N-phenyl-2-phenylquinolinium perchlorate

To a clear solution of phenylmagnesium bromide (prepared from bromobenzene (15.7 g, 0.1 mol) and magnesium (2.4 g, 0.1 mol) N-phenylquinolinium perchlorate (7 g, 0.023 mol) was added in powder form. The resulting homogeneous solution was cooled and an alcoholic solution of iodine (0.023 mol) and sodium acetate (0.023 mol) was added with stirring. After evaporation of the solvent the resulting solid was boiled in water (ca. 500 ml) and hot filtered. On cooling the filtrate in ice; a solid precipitated which was filtered off and recrystallised from aqueous
ethanol (yield 2.5 g, 20%)

m.p. 227-228°C  Literature reports 226°C, 172 222°C 173
N.m.r. (CDCl₃) δ 7.09-9.28 (m, aromatic-H)

2.9  Synthesis of Possible Metabolites of Quinolinium and Related Cations

The quinolones and related compounds prepared below were characterised by a combination of their u.v., infrared, n.m.r. and mass spectra which are given in the relevant sections in Chapter 3.

2.9.1  Preparation of N-methyl-2-quinolone 174

Dimethyl sulphate (5 g, 0.04 mol) was gradually added to quinoline (5 g, 0.039 mol), an exothermic reaction ensued which required cooling with water. The solid produced was dissolved in water, potassium ferri-cyanide (30 g) was added and the total volume of the solution was made up to 250 ml with water and transferred to a separating funnel to which 200 ml of ether was added. Potassium hydroxide (10%) was added in small quantities with shaking until the reaction mixture was strongly alkaline. The ethereal solution was separated and the aqueous alkaline solution was extracted twice more with ether (2 x 200 ml). The combined ether extracts were washed, dried over MgSO₄ and evaporated. The oil thus obtained was triturated with ether and put in ice to yield a solid which was recrystallised from 40/60 petroleum ether (yield 1.9 g, 31%).

m.p. 71-72°C  Literature reports 74°C, 174 72°C 175

2.9.2  Preparation of N-methyl-4-quinolone (Echinopsine) 176

To a solution of 4-quinolone (2 g, 0.01 mol) dissolved in methanol (25 ml) was added sodium hydroxide (3 ml, 5 M) and dimethyl sulphate (2 ml). The solution was filtered and the filtrate evaporated under reduced pressure. The solid obtained was dissolved in water (15 ml) and extracted with
chloroform (3 x 20 ml). The extracts were washed, dried over MgSO₄ and then evaporated to yield a pale yellow powder which was recrystallised from methanol (yield 1.75 g, 79%)

m.p. 150°C  Literature reports 152°C¹⁷⁶

2.9.3 Preparation of N-phenyl-2-quinolone

N,N-Diphenylcinnamalimide.¹⁷⁷  Cinnamoyl chloride (5 g, 0.03 mol) in benzene (20 ml) was added dropwise to a solution of diphenylamine (5 g, 0.03 mol) containing pyridine (4.7 g) at 5-10°C with stirring. The precipitated solid was filtered and washed with water and dilute hydrochloric acid and then recrystallised from ethanol to yield white needle-like crystals (yield 5 g, 55%)

m.p. 157-158°C  Literature reports 159-160°C,¹⁷⁷ 156°C¹⁷⁸

N-Phenyl-2-quinolone.¹⁷⁷  The cinnamalimide (1 g, 3.3 mmol) in dry chlorobenzene (30 ml) was mixed with anhydrous aluminium chloride (4 g) and heated on a steam bath. The reaction was monitored using t.l.c. [silica G₂₅₄ plates ran in isoamyl acetate, Rf value for the starting material is 0.72 and for N-phenyl-2-quinolone is 0.39]. The reaction was stopped at the disappearance of the starting material (after ca. 3 h) by treating with ice and dilute hydrochloric acid. The solution was extracted with dichloromethane; the extracts were washed with water, dried over MgSO₄ and evaporated to yield a brown oil. Trituration of the oil in 40/60 petroleum ether and cooling produced an off-white solid which was filtered. The solid was boiled in ethanol with charcoal and filtered hot. A white crystalline solid was obtained from the cooled filtrate (yield 0.4 g, 54%)

m.p. 137-139°C  Literature reports 142-3°C,¹⁷⁷ 138°C,¹⁷⁹ 137-139°C¹⁸⁰

2.9.4 Preparation of N-phenyl-4-quinolone

Diphenyl-2-cyanoethylamine.¹⁸¹  A mixture of diphenylamine (40 g,
0.236 mol) vinyl cyanide (18 ml, 0.472 mol) copper acetate (2 g) dissolved in butyric acid (18 ml) and fine copper powder (4 g) was refluxed and stirred for 12 h. Distillation of the crude product at atmospheric pressure removed the unchanged nitrile and butyric acid. Further distillation under vacuum gave a considerable fraction of unchanged diphenylamine, followed by the crude diphenyl-2-cyanoethylamine (b.p. 156-162°C/0.1-0.2 mm) yield 9 g.

β-Diphenylaminopropionic acid. To a solution of the above nitrile (9 g) in ethanol (100 ml) was added potassium hydroxide (20 g in 130 ml water) which was then refluxed for 4 h. The ethanol was then removed by distillation and the unchanged diphenylamine (this is present in the crude preparation of diphenyl-2-cyanoethylamine), which crystallises on cooling, filtered off. The filtrate was then acidified and the precipitated acid collected (7 g)

m.p. 105-106°C Literature reports 111-112°C

Infrared spectrum (KBr) ν_{CO} 1700 cm^{-1}

Mass spectrum: m/e 241 (M)

N-Phenyl-1,2,3,4-tetrahydro-4-quinolone. Phosphoric anhydride (5 g) was added to a solution of the above acid (4 g, 0.017 mol) in warm xylene (50 ml), which was then refluxed for 2 h. The xylene was then removed by steam distillation and the cold aqueous residue treated with an excess of sodium carbonate and extracted with benzene. The solvent was removed from the dried filtered extract and the residue on distillation furnished a pale yellow oil (b.p. 150-175°C/0.2 mm) which solidified and was recrystallised from ethanol (yield 1.4 g, 32%).

m.p. 75-77°C Literature reports 84°C 76-81°C

Mass spectrum: m/e 223 (M), 195 (M-CO)

N-Phenyl-4-quinolone. N-Phenyl-1,2,3,4-tetrahydro-4-quinolone (0.5 g, 2.2 mmol) and palladised charcoal (0.25 g) were refluxed in
ethanediol (20 ml). The progress of the reaction was monitored using t.l.c. [silica G254 plates ran in isoamyl acetate, Rf value for the starting material is 0.39 and for the required product is 0.11]. After 30 min the solution was filtered and the residual catalyst washed with hot solvent. A red oil was obtained on evaporation of the combined filtrate and washings under reduced pressure. Water was added to this oil and the N-phenyl-4-quinolone extracted with ether. The ether extracts were washed, dried over MgSO4 and evaporated to yield a white solid (0.27 g, 54%)

m.p. 125-126°C

Mass spectrum: m/e 221 (M), 193 (M-CO)

Infrared spectrum (KBr): νCO 1630 cm⁻¹

N.m.r. (CDCl3) δ 6.37 (d, C3-H), 7.00 (m, C2-H), 7.27-7.68 (m, C-H aromatic), 8.47 (m, C5-H)

No previous reports of the preparation of this compound were found in the literature, although its picrate salt has been prepared. Further confirmation of the identity of the product was afforded by elemental analysis

Analysis: C, 81.40; H, 5.10; N, 6.25

C15H11NO requires: C, 81.45; H, 4.97; N, 6.33

2.9.5 Preparation of N-methyl-7,8-benzo-2-quinolone

Potassium ferricyanide (3 g, 9 mmol) was added to a solution of N-methyl-7,8-benzo-2-quinolinium iodide (0.5 g, 1.55 mmol) in hot water. When the solution had cooled to room temperature potassium hydroxide was added until the mixture was strongly alkaline. This was then extracted with ether; the combined extracts were washed, dried over MgSO4 and evaporated to yield a solid. This was recrystallised from methanol to give yellow needle-like crystals (0.3 g, 33%)

m.p. 173°C Literature reports 174°C
2.9.6 Preparation of N-methyl-5,6-benzo-2-quinolone

This compound was obtained from N-methyl-5,6-benzoquinolinium by the method described in section 2.9.5. The evaporated ether extracts yielded a brown solid; this was recrystallised from aqueous ethanol to give golden platelets.

m.p. 178-180°C  Literature reports 182°C\(^{175}\)

2.9.7 Preparation of N-methyl-4-phenyl-2-quinolone\(^{183}\)

This compound was obtained from N-methyl-4-phenylquinolinium iodide (0.1 g, 0.29 mmol) by the method described in section 2.9.5; only a small amount of the required product was obtained.

m.p. 138-139°C  Literature reports 141-142°C\(^{184}\)

2.9.8 Preparation of N-methylphenanthridone\(^{175}\)

Phenanthridine (1.5 g, 0.008 mol) was added to dimethyl sulphate (5 ml, 0.052 mol) and heated on a steam bath for 30 min. The mixture was then cooled to ca. 50°C and potassium hydroxide (2.95 g in 100 ml of water) added. After disappearance of the two layers the mixture was neutralised by further addition of potassium hydroxide solution (0.5 M) and placed on an ice-bath. \(p\)-Benzoquinone (0.99 g, 0.008 mol) and then aqueous potassium hydroxide (0.5 g in 100 ml of water) were gradually added to the stirred mixture which was then allowed to reach room temperature. The mixture was extracted with ether (3 x 100 ml), the combined extracts were washed and dried over MgSO\(_4\) and the ether evaporated. The solid obtained was recrystallised from aqueous ethanol (yield 1.2 g, 68%)

m.p. 106-107°C  Literature reports 107-108°C\(^{175}\)

2.9.9 Preparation of N-methyl-4-methyl-2-quinolone\(^{185}\)

4-Methyl-2-quinolone (0.88 g, 5.5 mmol) was dissolved in 5% aqueous sodium hydroxide solution (45 ml) by heating to ca. 80°C. Dimethyl
sulphate was then added in small portions to the well stirred, hot solution until pH 3-4 was obtained. Upon cooling, the solution yielded crystals of the required product (0.65 g, 68%).

m.p. 128.5-129.5°C Literature reports 128-129°C, 185 130-132°C186

2.9.10 Preparation of N-methyl-2-methyl-4-quinolone187

To a solution of potassium hydroxide (0.71 g, 0.013 mol) in hot methanol (50 ml), 2-methyl-4-quinolone (2 g, 0.013 mol) was added. The methanol was removed and dimethyl sulphate (5 ml) added to the residue. The reaction mixture was heated on a steam bath for 30 min and then taken up in potassium hydroxide (2.5 M, 100 ml). The resulting purple solution was extracted with chloroform. The combined extracts were evaporated down, and the residual oil was redissolved in the minimum of chloroform and then passed through a column containing 50 g of aluminium oxide G. The eluate was evaporated to dryness and then taken up in the minimum of chloroform and upon addition of petroleum ether (40/60) an orange solid precipitated. This solid was sublimed at 120°C/0.03 mm, and the white crystals thus obtained were recrystallised from benzene (yield 0.65 g, 30%).

m.p. 178°C Literature reports 179.5-180.5°C187

2.9.11 Preparation of N-methyl-4-quinolone-2-carboxylic acid188

A mixture of 4-quinolone-2-carboxylic acid (0.05 g, 2.64 mmol), sodium hydride (0.33 g, 8 mmol; 58% in mineral oil) and dry DMF (15 ml) was stirred under a nitrogen atmosphere for 30 min at 80°C. The solution was then cooled to room temperature and iodomethane (2.5 ml) added. After being stirred for 2 h, an additional 2.5 ml of iodomethane was added and the solution stirred for a further 2 h. The solvent was removed and the residue recrystallised from water to give white needles of the methyl ester of the required product, m.p. 143°C (literature reports 145-146°C188).
This was refluxed in a 10% aqueous sodium hydroxide solution (5 ml) and ethanol (0.5 ml) for 2 h and then acidified. The solution was left in the refrigerator overnight and the precipitated acid was filtered (yield 0.08 g, 15%).

m.p. 186-187°C  Literature reports 186-187°C¹⁸⁸

2.9.12 Preparation of 3-methyl-4-quinazolinone¹⁸⁹

4-Quinazolinone (5 g, 0.034 mol) was dissolved in methanol (ca. 50 ml) containing potassium hydroxide (2 g, 0.05 mol). Iodomethane (5 g, 0.035 mol) was added and the mixture left to stand. After 2 h white crystals had precipitated out of solution; these were filtered and dissolved in the minimum amount of cold water. After ca. 30 min crystals separated out again and were recrystallised from water giving colourless needles, which were dried in a vacuum oven at 60°C.

m.p. 105°C  Literature reports 105°C¹⁸⁹

N.m.r. (DMSO-d₆) δ 3.52 (N₃-CH₃)

Infrared spectrum (KBr) ν 1670 cm⁻¹

2.9.13 Preparation of 3-methyl-2,4-quinazolinedione¹⁹⁰

2,4-Quinazolinedione (8 g, 0.05 mol) and sodium hydroxide (2 g, 0.05 mol) were dissolved in a 50% aqueous solution of methanol (250 ml). Iodomethane (7 g, 0.05 mol) was added and the solution refluxed for 2.5 h. This solution after hot filtration and cooling, deposited white crystals which were filtered off and then dissolved in 0.7 M sodium hydroxide. This solution was filtered and the filtrate acidified to yield an amorphous precipitate which was recrystallised from methanol to yield white needles.

m.p. 237-239°C  Literature reports 237-239°C,¹⁹⁰ 241°C¹⁹¹

2.10 Discussion of Synthetic Methods

All N-heterocyclic quaternary compounds tested for substrate activity
with aldehyde oxidase were synthesised by the author. In order to confirm the identity of oxidation products formed as a result of enzymatic action, authentic samples of the suspected products were also synthesised (where applicable) and used for comparison purposes.

Syntheses were generally carried out using literature methods. Products were characterised from their melting points and a combination of their u.v., infrared, n.m.r. and mass spectra. If the product had not been previously made then further confirmation of its identity was afforded by elemental analysis (performed by Elemental Micro-Analysis Ltd., Beaworthy, Devon, U.K.).

Some of the relevant factors pertaining to the syntheses described in the experimental section are briefly discussed below.

2.10.1 N-Methyl quaternary compounds

The majority of N-methyl quaternary compounds were obtained by refluxing the corresponding heterocyclic base with iodomethane in ethanol. More severe conditions were required to N-methylate 7,8-benzoquinoline due to the steric hindrance of the nitrogen in this compound. Factors influencing quaternisation of N-heterocycles have been reviewed by Duffin\textsuperscript{192} and Zoltewicz and Deady.\textsuperscript{193}

The iodide salts were converted to the chlorides by using an ion-exchange column or by refluxing with silver chloride. The perchlorate salts were formed by adding sodium perchlorate to an aqueous solution of the iodide. N.m.r. spectroscopy was particularly useful for establishing that the N-methylation had been successful since the chemical shift of the N-CH\textsubscript{3} is characteristic.

The N-heterocycles used in the N-methylation reaction were available commercially except for 4-phenyl quinoline which was synthesised by the method of Kenner and Stathan;\textsuperscript{158} this involved condensation of
aniline and β-chloropropiophenone. The required product was isolated as the picrate salt in low yield and the free base was obtained by dissolving the salt in lithium hydroxide and extracting with ether.\textsuperscript{161}

\[
\begin{align*}
\text{Aniline} + \text{β-Chloropropiophenone} & \rightarrow \text{Quinazoline}
\end{align*}
\]

\textit{N-Methyl-2-phenylquinolinium iodide} was obtained in excellent yield by the Grignard reaction of \textit{N}-methylquinolinium iodide and phenylmagnesium bromide, followed by iodine/sodium acetate oxidation.\textsuperscript{163} This compound can also be prepared from 2-phenylquinoline and iodomethane.\textsuperscript{164} The advantage of the method used was that the starting material was already available whereas 2-phenylquinoline would have required synthesis. The perchlorate salt was obtained from the iodide as described above.

\textit{3-Methyl-2-oxoquinazolinium chloride}. The overall reaction sequence for the synthesis of this compound as described by Parg and Hamprecht\textsuperscript{167} is illustrated below:

\[
\begin{align*}
\text{Quinazolin-2-one} & \xrightarrow{\text{i}} \text{Quinazolin-2-one-N-oxide} \\
\text{Quinazolin-2-one-N-oxide} & \xrightarrow{\text{ii}} \text{Quinazolin-2-one-N-oxide-N-methyl-N-oxide} \\
\text{Quinazolin-2-one-N-oxide-N-methyl-N-oxide} & \xrightarrow{\text{iii}} \text{Quinazolin-2-one-N-oxide-N-methyl-N-oxide-N-methyl-N-oxide-N-oxide} \\
(i) O-Nitrobenzaldehyde dimethylacetal was obtained in excellent yield from the interaction of o-nitrobenzaldehyde with the methoxylating agent, dimethyl sulphite. (ii) The nitro group of this acetal was reduced with an acidic aqueous solution of sodium sulphide, the resulting product is not very stable since it can be readily converted to the free aldehyde and thus was used immediately in the following step. (iii) Interaction with methylisocyanate at room temperature gave a very good yield of the urea, which in hydrochloric acid at 80°C [(iv)] readily ring closed to form 3-methyl-2-oxoquinazolinium chloride. Melting point, infrared and n.m.r. spectra confirmed the identity of the final product.

The iodide salt of this cation was prepared from interaction of 2-hydroxyquinazoline (kindly donated by C. Stubley of Bradford University) with iodomethane but the yield was poor (13%).

3-Methyl-2-oxoquinazolinium chloride was dissolved in water and the solution neutralised with sodium hydroxide; the pseudobase 3,4-dihydro-4-hydroxy-3-methyl-2-quinazolinone, precipitated.

This reaction and its importance is more fully discussed in Chapter 5.

N-Methylation of quinazoline with iodomethane occurs predominately on the N-3 atom. The salt thus produced forms the pseudobase, 3,4-dihydro-4-hydroxy-3-methylquinazoline, in alkali.
The stability of this pseudobase is comparable to the stability of 3,4-dihydroquinazoline and is another illustration of the marked tendency for position 3,4 in quinazoline to acquire the dihydro structure.\textsuperscript{169}

### 2.10.2 N-Phenyl quaternary compounds

\textit{N-Phenylquinolinium perchlorate} was obtained by Skraup synthesis between diphenylamine and acrolein, under the conditions described by Mortelmans and Van Binst.\textsuperscript{172} Grignard reaction of this cation with phenylmagnesium bromide, immediately followed by iodine/sodium acetate oxidation yielded \textit{N-phenyl-2-phenylquinolinium perchlorate}\textsuperscript{172,173}

\begin{align*}
\text{PhNH} & + \text{CH}_2=\text{CHCHO} \overset{\text{HCl}}{\rightarrow} \text{PhN}^+\text{C}_6\text{H}_5\text{ClO}_4^- \\
& + \text{PhMgBr} \rightarrow \text{PhN}^+\text{C}_6\text{H}_5 \text{ClO}_4^- \\
& + \text{I}_2/\text{NaOAc} \rightarrow \text{PhN}^+\text{C}_6\text{H}_5 \text{ClO}_4^-
\end{align*}

It is interesting to note that the Grignard reagent attacked C-2 rather than C-4 (which is also susceptible to nucleophilic attack) despite the former carbon being much more sterically hindered.
2.10.3 2-Quinolones

*N*-Methyl-2-quinolone, *N*-methyl-5,6-benzo-2-quinolone, *N*-methyl-7,8-benzo-2-quinolone and *N*-methyl-4-phenyl-2-quinolone were prepared by the oxidation of the corresponding *N*-methyl quaternary compound with potassium ferricyanide in strong alkaline solution; the oxidation product then being extracted with ether. This is a long established synthetic procedure and is known as the Decker oxidation. The reaction is considered to involve formation of a pseudobase from the *N*-heterocyclic cation in very basic conditions, which is then oxidised by ferricyanide (see Chapter 5).

*N*-Methylphenanthridone was similarly prepared except *p*-benzoquinone was used as the oxidant in place of ferricyanide according to the method of Hase. 175

*N*-Methyl-4-methyl-2-quinolone. Decker oxidation of *N*-methyl-4-methylquinolinium has been reported to give *N*-methyl-4-methyl-2-quinolone, but only in low yield (7.5%)194. In alkaline conditions the cation can, in addition to forming the pseudobase, lose a proton to produce a methylene base which is highly reactive and probably accounts for the low yield of the required quinolone.

![Methylene Base](image)

An alternative preparation reported by Nadzan and Rinehart,164 which gives a better yield of *N*-methyl-4-methyl-2-quinolone, was thus adopted. This
involved N-methylation of 4-methyl-2-quinolone using dimethyl sulphate in alkaline conditions. This general procedure has been used to obtain N-methyl-2-quinolone \(^{195}\) and also various N-methyl-4-quinolones (see section 2.10.4).

\(N\)-Phenyl-2-quinolone \(^{177}\) was conveniently prepared in reasonable yield from diphenylamine and cinnamoyl chloride, followed by treatment with anhydrous aluminium chloride as illustrated:

\[\text{NH} + \text{Cl}/\text{C} \overset{i}{\text{O}} /\text{I} /\text{NO} \rightarrow \text{AlCl}_3 \rightarrow \]

The mechanism whereby aluminium chloride catalyses ring closure and dearylation proposed by Manimaran and Ramakrishnan, is depicted over. The 4-aryl-3,4-dihydro species (3) is considered to be an intermediate which is formed via (2) either by intramolecular Friedel Crafts alkylation or acid-catalysed Michael addition of (1). Aluminium chloride forms a \(\pi\)-complex as in (3) which effects the rupture of the \(C_4\)-aryl bond and thus catalyses dearylation.
N-Methyl-4-quinolone was produced in good yield by the N-methylation of 4-quinolone using dimethyl sulphate in alkaline conditions.
The ultimate formation of the N-methylated rather than the O-methylated product in this type of reaction and the greater thermodynamic stability of N-methyl-4-quinolone compared with 4-methoxyquinoline has been discussed in the literature.¹⁹⁶-¹⁹⁸

*N-Methyl-2-methyl-4-quinolone* was similarly synthesised from 2-methyl-4-quinolone following the procedure outlined by Werny and Scheuer;¹⁸⁷ except that instead of refluxing with dimethylsulphate (which appeared to decompose the starting material) the reaction mixture was heated over a steam-bath. The extracted material required chromatography, sublimation and recrystallisation to obtain the required product in a pure form and the yield was higher than that reported by Werny and Scheuer.

*N-Methyl-4-quinolone-2-carboxylic acid.* Interaction of dimethyl sulphate with 4-quinolone-2-carboxylic acid produced the methyl ester of the required product which was subsequently hydrolysed to give the acid.

*N-Phenyl-4-quinolone.* The synthesis of this compound from diphenylamine involved four stages as illustrated below:

![Chemical diagram](image)
(i) Cyanoethylation of diphenylamine was carried out using the method of Cookson and Mann, except that instead of heating the reaction components in acetic acid at 150°C in an autoclave, butyric acid was used in lieu of acetic acid and the reaction mixture refluxed. This modification did not appear to affect the yield of the required product. Finely divided copper acted as a catalyst in this reaction. (ii) Hydrolysis of the nitrile compound from stage (i) in alkali, furnished \( \beta \)-diphenylamino-propionic acid. (iii) This acid when boiled in xylene with phosphoric anhydride ring closed with elimination of water to yield N-phenyl-1,2,3,4-tetrahydro-4-quinolone. Alternatively this product could have been obtained by treating the acid with phosphorus pentachloride and aluminium chloride or zinc chloride in acetic anhydride. (iv) Dehydrogenation of the tetrahydroquinolone was achieved by refluxing with palladised charcoal in ethanediol, the progress of the reaction being monitored by t.l.c. The product was isolated in reasonable yield (54\%) and the elemental analysis was consistent with it being N-phenyl-4-quinolone (C\(_{15}\)H\(_{11}\)NO). The mass spectrum for this compound had a molecular ion at m/e 221 compared with m/e 223 for the starting material, indicating the dehydrogenation reaction to have been successful. This was also confirmed by the n.m.r. spectrum since only olefinic and aromatic resonances were observed. The infrared spectrum was characterised by a peak in the carbonyl stretching region at a frequency (1630 cm\(^{-1}\)) typical for a 4-quinolone (all spectral data are given in section 2.9.4). Thus the product obtained has been identified as N-phenyl-4-quinolone; previously only the picrate salt of this compound had been prepared.

Spectral analysis for characterising and differentiating 2-quinolones and 4-quinolones is discussed in section 3.1.
2.10.5 Quinazolinones

3-Methyl-4-quinazolinone was prepared by the N-methylation of 4-quinazolinone using iodomethane in alkali.\footnote{189} Under these conditions N-3 methylation is known to be favoured over N-1 methylation. 3-Methyl-2,4-quinazolinedione was similarly prepared from 2,4-quinazolinedione.\footnote{190}
CHAPTER 3

Identification of Oxidation Products Resulting from
Incubation of Quinolinium and Related
Cations with Aldehyde Oxidase

3.1 Introduction

Product identification is an important aspect of enzyme studies, particularly with an enzyme as versatile as aldehyde oxidase. Techniques employed must be very sensitive because of the small quantities of material obtained, and in this respect ultraviolet (u.v.) spectroscopy has been widely used. However u.v. spectra give only limited information about the structure of a compound, furthermore it is often difficult to interpret spectra when more than one product is formed, with the possibility of minor products being overlooked. In this present study products resulting from incubation of quinolinium and related cations with aldehyde oxidase were generally characterised by a combination of chromatographic, infrared, nuclear magnetic resonance and mass spectral data, which was compared with that obtained for an authentic sample of the suspected product.

Incubations were carried out at pH 7 using partially purified preparations of aldehyde oxidase from either rabbit or guinea pig and extracted as described in section 2.4.1. The extracts were used to determine $R_f$ values on thin layer plates; high pressure liquid chromatography (h.p.l.c.) was employed to separate incubation products and obtain retention volume data. Fractions corresponding to these products were collected from the h.p.l.c. eluate and evaporated to dryness. Clearly resolved infrared, u.v. and mass spectra were obtained from the residue.

Nuclear magnetic resonance (n.m.r.) is a very powerful technique for the determination of the structures of organic compounds. Until
recently comparatively large samples (~10 mg) were necessary to obtain spectra with satisfactory signal:noise ratio and hence examination of products resulting from enzyme catalysed reactions by n.m.r. was impracticable. However, the advent of Fourier Transform n.m.r. has greatly improved the sensitivity and it is now possible to obtain spectra with samples ~50 μg. Where possible, n.m.r. spectra were measured of products extracted from large scale incubations [see section 2.4.1(ii)] and isolated using preparative chromatography plates.

Aldehyde oxidase catalysed oxidation of N-heterocycles usually occurs at a carbon atom α to the ring nitrogen, i.e. C-2 in quinoline; however in this present study of various quinolinium cations, the possibility of oxidation at a carbon γ to the ring nitrogen i.e. C-4 was also considered. The use of spectral data to distinguish and characterise 2- and 4-quinolones has been well documented\(^{201,202}\) and is briefly discussed below.

**U.v. spectra.** 2-quinolones absorb strongly at ~270 nm whereas the 4-quinolones have a minimum at this wavelength.\(^{202,203}\) Furthermore 4-quinolones often show an unexplained splitting of the near ultraviolet maximum into two peaks (e.g. \(\lambda_{\text{max}}\) for N-methyl-4-quinolone occurs at 336 and 323 nm).\(^{204}\) Characteristically in acid solution the \(\lambda_{\text{max}}\) for 4-quinolones are shifted whereas acid has no effect on the spectra for 2-quinolones.\(^{202}\) This difference is due to the much greater basicity of 4-quinolones which can be regarded as vinylogous amides and are protonated in acid thus:

\[
\begin{align*}
\text{CH}_3 & \quad \overset{H^+}{\longrightarrow} \quad \text{CH}_3 \\
\end{align*}
\]
**N.m.r. spectra.** Application of a magnetic field can cause circulations of the electrons within a functional group but the circulation may be more facile in one plane than another. Thus chemical bonds are magnetically anisotropic; the anisotropy of the carbonyl group causes deshielding (−) of protons lying in a cone extending from the carbonyl oxygen atom but shielding (+) of protons lying outside this cone.\(^{205}\)

![Diagram of a cone with positive and negative charges]

The C-5 proton of the 4-quinolones characteristically resonates at low field 7.93-8.46 p.p.m. as a result of deshielding by the peri-carbonyl group at C-4.\(^{201}\)

**Infrared spectra.** Generally the carbonyl absorption of 2-quinolones occurs in the 1660-1650 cm\(^{-1}\) region whereas the 4-quinolones absorb at lower frequency in the 1630-1620 cm\(^{-1}\) range;\(^{203,206,207}\) this was found to be true for the authentic quinolones synthesized in this present study. However, some quinolones of both types absorb in the intermediate 1645-1630 cm\(^{-1}\) region, these can still be distinguished since the intensity of the carbonyl band in the 2-quinolones is much greater than in the 4-quinolones.\(^{208}\)

**Mass spectra.** Although both 2- and 4-quinolones break down similarly in the mass spectrometer by initial loss of the CO fragment, isomeric species were found to be distinguishable from the relative peak heights.
Using a combination of the above spectroscopic techniques one can confidently assign the structure.

All of the N-heterocyclic cations and the majority of the suspected oxidation products were synthesised (where applicable) by the author (see sections 2.8 and 2.9) to provide authentic samples for comparison purposes.

3.2 Oxidation Products of N-Methylquinolinium Salts

Preliminary analysis by t.l.c. of the extracts resulting from the incubation of N-methylquinolinium salts with partially purified aldehyde oxidase from either rabbit or guinea pig livers indicated the formation of two products. The chromatographic data of these metabolites was compared with that obtained from authentic samples of the possible oxidation products.

T.l.c. data

<table>
<thead>
<tr>
<th></th>
<th>R&lt;sub&gt;f&lt;/sub&gt; x 100</th>
<th>Detection Tests</th>
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<tr>
<td></td>
<td>Solvent A</td>
<td>Solvent B</td>
</tr>
<tr>
<td>N-Methylquinolinium+ X&lt;sup&gt;-&lt;/sup&gt;</td>
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<td>s</td>
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<tr>
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<td>86</td>
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<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>N-Methyl-4-quinolone</td>
<td>31</td>
<td>34</td>
</tr>
</tbody>
</table>

Conditions as described in section 2.5.1. X = Cl, I, ClO<sub>4</sub>. s = streaking from baseline. * = faint spot.
**H.p.l.c. data**

<table>
<thead>
<tr>
<th></th>
<th>Retention Volumes/ml</th>
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<tr>
<td></td>
<td>( a_1^+ )</td>
</tr>
<tr>
<td>N-Methylquinolinium+X^-</td>
<td>n</td>
</tr>
<tr>
<td>Metabolite 1</td>
<td>4.5</td>
</tr>
<tr>
<td>Metabolite 2</td>
<td>7.5</td>
</tr>
<tr>
<td>N-Methyl-2-quinolone</td>
<td>4.5</td>
</tr>
<tr>
<td>N-Methyl-4-quinolone</td>
<td>7.5</td>
</tr>
</tbody>
</table>

Conditions as described in section 2.5.2. \( n \) = not eluted. \( v \) = variable, initially comes off with solvent front but after continual use eventually compound is not eluted. * see Figure 3.5.

N-Methylquinolinium iodide, chloride and perchlorate were each incubated with aldehyde oxidase and it appears that the type of anion has no effect on formation of either product.

Eluates corresponding to the observed peaks in the adsorption h.p.l.c. system \( a_1 \) were collected and evaporated down to dryness. Residues obtained in this manner were subjected to infrared and mass spectroscopy.

As shown in Figure 3.1 the infrared spectrum of metabolite 1 is characterised by a band in the carbonyl stretching region at 1650 cm\(^{-1}\) and is identical to that obtained for N-methyl-2-quinolone. The spectrum of metabolite 2 is also characterised by a carbonyl stretching band, but at lower frequency 1620 cm\(^{-1}\) and corresponds to that for N-methyl-4-quinolone.

The mass spectra for both incubation products have a molecular ion peak at m/e 159 and the breakdown pattern for both is consistent with initial loss of CO. The spectra are however distinguishable by the relative intensities of the peaks (see Figure 3.2). The mass spectra for metabolites 1 and 2 are the same as those obtained for N-methyl-2-quinolone and
Figure 3.1. Infrared spectra of N-methyl-2-quinolone, N-methyl-4-quinolone and the incubation products of N-methylquinolinium.
N-methyl-4-quinolone respectively, the initial fragmentation for which are shown below.\(^{209}\)

![Chemical structures](image)

The identity of the two products was further confirmed from the u.v. spectral data.

**U.v. spectral data**

<table>
<thead>
<tr>
<th></th>
<th>(\lambda_{\text{max}}/\text{nm}) (i)</th>
<th>(ii)</th>
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<tbody>
<tr>
<td>N-Methylquinolinium(^+) (\text{ClO}_4^-)</td>
<td>316</td>
<td>no change</td>
</tr>
<tr>
<td>Metabolite 1</td>
<td>326,270</td>
<td>no change</td>
</tr>
<tr>
<td>Metabolite 2</td>
<td>336,323</td>
<td>308</td>
</tr>
<tr>
<td>N-Methyl-2-quinolone</td>
<td>326,270</td>
<td>no change</td>
</tr>
<tr>
<td>N-Methyl-4-quinolone</td>
<td>336,323</td>
<td>308</td>
</tr>
</tbody>
</table>

(i) Measured in solvent from h.p.l.c. system p\(_5\).  (ii) Spectra after adjustment to pH 1 with HCl.
Figure 3.2. Mass spectra of N-methyl-2-quinolone, N-methyl-4-quinolone and the incubation products of N-methylquinolinium
Metabolites 1 and 2 were isolated from large scale incubations using preparative t.l.c. with solvent A as the mobile phase and their n.m.r. spectra measured (see experimental for details, section 2.5.6). The spectrum for metabolite 1 was found to be identical to that for N-methyl-2-quinolone (see Figure 3.3) both having characteristic resonances at $\delta$ 3.72 (s, N-CH$_3$) and 6.71 (d, C$_3$-H; J$_{(3,4)}$ 9.52 Hz). The n.m.r. spectrum of metabolite 2 was readily distinguishable from metabolite 1 and corresponded to that for N-methyl-4-quinolone (see Figure 3.4), with peaks at $\delta$ 3.79 (s, N-CH$_3$), 6.24 (d, C$_3$-H; J$_{(3,4)}$ 9.52 Hz) and 8.45 (m, C$_5$-H). The low field resonance of the C-5 proton is particularly characteristic of a 4-quinolone, as previously mentioned.

The identities of the two incubation products have been unequivocally established as N-methyl-2-quinolone and N-methyl-4-quinolone. Therefore aldehyde oxidase catalysed oxidation of N-methylquinolinium must also occur at C-4 and not only at C-2 as previously reported.$^{3,31}$ The ratio of 2- to 4-quinolone formation was found to depend on the source of aldehyde oxidase used, as shown in Figure 3.5.
Figure 3.3. N.m.r. spectra of N-methyl-2-quinolone and one of the incubation products of N-methylquinolinium

N-Methyl-2-quinolone

Metabolite 1
Figure 3.4. N.m.r. spectra of N-methyl-4-quinolone and one of the incubation products of N-methylquinolinium

N-Methyl-4-quinolone

Metabolite 2

* CHCl₃ impurity in CDCl₃
Figure 3.5. H.p.l.c. traces of the incubation products of N-methyl-quinolininium perchlorate

(i) with rabbit enzyme                      (ii) with guinea pig enzyme

\[
\text{RATIO} \quad 2:4 \text{ Quinolones} \quad 19 \pm 2 \quad 10 \pm 1
\]

* H.p.l.c. system a

The proportion of 4-quinolone formed is twice as great with aldehyde oxidase derived from guinea pig than from rabbit; however it still remains the minor product.

When N-methyl-2-quinolone or N-methyl-4-quinolone were incubated with enzyme, quantitative recovery of the starting material was obtained and thus neither of these compounds are further metabolised by aldehyde oxidase.
3.3 Oxidation Products of N-Phenylquinolinium Perchlorate

In view of the results obtained for the N-methylquinolinium cation, it was of interest to examine the effect on product formation of a bulkier nitrogen substituent, e.g. phenyl. Incubation of N-phenylquinolinium perchlorate with aldehyde oxidase derived from either rabbit or guinea pig yielded two products. The chromatographic data of these metabolites were compared with those obtained from authentic samples of the anticipated products. N-Phenyl-4-quinolone had not been previously synthesised as the free base. However this compound was obtained in the present study using a modification of the procedure designed to yield the picrate salt (see section 2.9.4).

T.l.c. data

<table>
<thead>
<tr>
<th></th>
<th>Solvent C</th>
<th>Solvent D</th>
<th>Solvent E</th>
<th>Detection Tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Phenylquinolinium$^+\text{ClO}_4^-$</td>
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<td>s</td>
<td>0</td>
<td>purple +</td>
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<tr>
<td>Metabolite 1</td>
<td>39</td>
<td>76</td>
<td>39</td>
<td>purple +</td>
</tr>
<tr>
<td>Metabolite 2</td>
<td>11</td>
<td>60</td>
<td>23</td>
<td>yellow +</td>
</tr>
<tr>
<td>N-Phenyl-2-quinolone</td>
<td>39</td>
<td>76</td>
<td>39</td>
<td>purple +</td>
</tr>
<tr>
<td>N-Phenyl-4-quinolone</td>
<td>11</td>
<td>60</td>
<td>23</td>
<td>yellow +</td>
</tr>
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</table>

Conditions as described in section 2.5.1
**H.p.l.c. data**

<table>
<thead>
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<th>Metabolite</th>
<th>a₄</th>
<th>p₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Phenylquinolinium⁺ ClO₄⁻</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>Metabolite 1</td>
<td>6.4</td>
<td>6.3</td>
</tr>
<tr>
<td>Metabolite 2</td>
<td>12.8</td>
<td>7.2</td>
</tr>
<tr>
<td>N-Phenyl-2-quinolone</td>
<td>6.4</td>
<td>6.3</td>
</tr>
<tr>
<td>N-Phenyl-4-quinolone</td>
<td>12.8</td>
<td>7.2</td>
</tr>
</tbody>
</table>

Conditions as described in section 2.5.2. See Figure 3.10

The infrared spectra of metabolites 1 and 2 exhibit carbonyl stretching bands at 1660 and 1630 cm⁻¹ respectively. The spectrum of metabolite 1 is identical to that obtained for N-phenyl-2-quinolone, whilst the spectrum of metabolite 2 corresponds to that of N-phenyl-4-quinolone (see Figure 3.6).

The mass spectra of N-phenyl-2-quinolone, N-phenyl-4-quinolone and both metabolites are compared in Figure 3.7. They all have a molecular ion peak of m/e 221 and although the fragmentation patterns are similar, it is the peak intensities which differentiate the spectra. The mass spectra for metabolites 1 and 2 were found to correspond to those for N-phenyl-2-quinolone and N-phenyl-4-quinolone respectively.

The identities of the two metabolites was further confirmed by their u.v. data.
Figure 3.6. Infrared spectra of N-phenyl-2-quinolone, N-phenyl-4-quinolone and the incubation products of N-phenylquinolinium.
Figure 3.7. Mass spectra of N-phenyl-2-quinolone, N-phenyl-4-quinolone and the incubation products of N-phenyllquinolinium.
**U.v. spectral data**

<table>
<thead>
<tr>
<th></th>
<th>(\lambda_{\text{max}}/\text{nm})</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Phenylquinolinium(^+) Cl(_4)^(-)</td>
<td>317  no change</td>
</tr>
<tr>
<td>Metabolite 1</td>
<td>326,273  no change</td>
</tr>
<tr>
<td>Metabolite 2</td>
<td>335,323  320</td>
</tr>
<tr>
<td>N-Phenyl-2-quinolone</td>
<td>326,273  no change</td>
</tr>
<tr>
<td>N-Phenyl-4-quinolone</td>
<td>335,323  320</td>
</tr>
</tbody>
</table>

(i) measured in solvent from h.p.l.c. system a\(_1\). (ii) spectra after adjustment to pH 1 with HCl.

Metabolites 1 and 2 were isolated from large scale incubations using preparative t.l.c. with solvent C as the mobile phase and their n.m.r. spectra measured and found to be readily distinguishable. On inspection, the spectrum for metabolite 1 was found to be identical to that for N-phenyl-2-quinolone (Figure 3.8) whilst the spectrum for metabolite 2 which is particularly characterized by the low field resonance at 88.47 corresponds to that for N-phenyl-4-quinolone (Figure 3.9).

From the above evidence N-phenyl-2-quinolone and N-phenyl-4-quinolone have been identified as oxidation products of N-phenylquinolinium perchlorate. The ratio of 2- to 4-quinolone formation was dramatically affected by the source of aldehyde oxidase used as shown in Figure 3.10.

Interestingly it was found that whilst the major metabolite is N-phenyl-2-quinolone with rabbit liver aldehyde oxidase, it is N-phenyl-4-quinolone with guinea pig enzyme.
Figure 3.8. N.m.r. spectra of N-phenyl-2-quinolone and one of the incubation products of N-phenylquinolinium

N-Phenyl-2-quinolone

Metabolite 1

* CHCl₃ impurity in CDCl₃
Figure 3.9. N.m.r. spectra of N-phenyl-4-quinolone and one of the incubation products of N-phenylquinolinium

N-Phenyl-4-quinolone

Metabolite 2

* CHCl₃ impurity in CDCl₃
Figure 3.10. H.p.l.c. traces of the incubation products of N-phenyl-quinolinium perchlorate

(i) with rabbit enzyme

(ii) with guinea pig enzyme

RATIO

4.7 ± 0.4

2:4 Quinolones

0.077 ± 0.0085

* H.p.l.c. system a₄

3.4 Identification of the Enzyme System

Controls were carried out to eliminate other possible enzymatic or non-enzymatic pathways of oxidation which might account for the hitherto unexpected simultaneous production of the 4-quinolones along with the 2-isomers.

Table 3.1 shows the result of purification of aldehyde oxidase, from crude liver slices to the highly purified DEAE stage, on the product
ratios using \( N \)-methylquinolinium perchlorate. The ratio was found to be constant (within experimental error) throughout the purification procedure. This eliminates the possibility of oxidation by the mono-oxygenase system, which is destroyed during the initial heating stage at \( 60^\circ C \). Any xanthine oxidase present is separated from the aldehyde oxidase at the DEAE step of purification. A further reason for discounting oxidation by xanthine oxidase was that incubation with bovine milk xanthine oxidase under the same conditions did not yield any significant amounts of products.

Incubations carried out in the presence of menadione, a potent inhibitor of aldehyde oxidase, produced neither 2- nor 4-quinolone. This is illustrated in Figure 3.11. The u.v. spectrum obtained when guinea pig aldehyde oxidase had been incubated for 30 minutes with \( N \)-phenyl-quinolinium perchlorate was predominantly that of the \( N \)-phenyl-4-quinolone. When this was repeated with menadione present no change in the spectrum was observed, i.e. no 4-quinolone was produced. The procedure was repeated with rabbit enzyme, where \( N \)-phenyl-2-quinolone is the major contributor to the final u.v. spectrum obtained and again menadione prevented the formation of 2-quinolone. From the above, it would appear that both compounds are genuine products of aldehyde oxidase catalysed oxidation.

Superoxide anions and hydrogen peroxide are produced in varying amounts when a substrate is oxidised by aldehyde oxidase under aerobic conditions. Incubations carried out with the substrate, \( N \)-methyl-quinolinium perchlorate, using partially purified rabbit enzyme, (a) in the presence of catalase (1 mg/5 ml) or (b) under anaerobic conditions with potassium ferricyanide (10\(^{-2}\) M) as the electron acceptor gave the same product ratios as in previous experiments. Therefore, removing peroxide as in (a) or preventing the formation of both superoxide and
Table 3.1. Ratio of 2:4 quinolone* production on enzyme purification

<table>
<thead>
<tr>
<th>Stage</th>
<th>Protein mg</th>
<th>Specific Activity (μmol/min per mg of protein)</th>
<th>Total Product Yield (%)</th>
<th>Ratio 2/4 ± (n = 6)</th>
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</thead>
<tbody>
<tr>
<td>Liver slices</td>
<td>200</td>
<td>---</td>
<td>28</td>
<td>17</td>
</tr>
<tr>
<td>Ammonium sulphate precipitation</td>
<td>29</td>
<td>0.275</td>
<td>78</td>
<td>18</td>
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<tr>
<td>G75-Sephadex</td>
<td>10</td>
<td>0.346</td>
<td>91</td>
<td>21</td>
</tr>
<tr>
<td>D.E.A.E. cellulose</td>
<td>0.45</td>
<td>2.84</td>
<td>12.5</td>
<td>17</td>
</tr>
</tbody>
</table>

* N-Methyl
Figure 3.11. U.v. spectra of incubation mixtures of N-phenylquinolinium perchlorate

(i) with rabbit enzyme

(a) --- $10^{-4}$ M solution of N-phenylquinolinium, phosphate buffer pH 7.

(b) As (a) plus aldehyde oxidase, incubated for 30 mins at 30°C.

(c) As (b) with $2 \times 10^{-5}$ M menadione

(ii) with guinea pig enzyme
peroxide (b) did not alter the amount of either product. Furthermore the results of incubation (b) indicate that the oxygen incorporated into both products is derived from water not oxygen. Both 2- and 4-quinolones are thus genuine products of aldehyde oxidase catalysed oxidation.

3.5 Oxidation Products of N-Methyl-7,8-benzoquinolinium Salts

Two products were detected by h.p.l.c. and t.l.c. on incubation of N-methyl-7,8-benzoquinolinium with aldehyde oxidase from either rabbit or guinea pig. From the foregoing, the two metabolites would be anticipated to be N-methyl-7,8-benzo-2-quinolone and N-methyl-7,8-benzo-4-quinolone; unfortunately there have been no reports in the literature of synthesis of the latter compound. Attempts to prepare the 4-quinolone by N-methylation of 4-hydroxy-7,8-benzoquinoline (a compound which itself proved difficult to synthesise210) were not successful. However, as previously stated, spectral data characterising other 4-quinolones has been well documented.

T.l.c. data

<table>
<thead>
<tr>
<th>Solvent A</th>
<th>Solvent F</th>
<th>R_f x 100</th>
<th>Detection Tests</th>
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<tbody>
<tr>
<td></td>
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<td>(i) 375 nm</td>
<td>(ii) 253.7 nm</td>
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<table>
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<th>R_f x 100</th>
<th>Detection Tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Methyl-7,8-benzoquinolinium+ X^-</td>
<td>s</td>
<td>blue</td>
</tr>
<tr>
<td>Metabolite 1</td>
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<td>65</td>
</tr>
<tr>
<td>Metabolite 2</td>
<td>59</td>
<td>31</td>
</tr>
<tr>
<td>N-Methyl-7,8-benzo-2-quinolone</td>
<td>75</td>
<td>65</td>
</tr>
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Conditions as described in section 2.5.1. X = Cl, ClO_4, I.
**H.p.l.c. data**

<table>
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<th>a₂</th>
<th>a₆</th>
<th>a₉</th>
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<tbody>
<tr>
<td>N-Methyl-7,8-benzoquinolinium⁺ ClO₄⁻</td>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>Metabolite 1</td>
<td>3.6</td>
<td>6</td>
<td>15.6</td>
</tr>
<tr>
<td>Metabolite 2</td>
<td>7.8</td>
<td>14</td>
<td>n</td>
</tr>
<tr>
<td>N-Methyl-7,8-benzo-2-quinolone</td>
<td>3.6</td>
<td>6</td>
<td>15.6</td>
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</table>

Conditions as described in section 2.5.2. *See Figure 3.15.*

Eluates corresponding to metabolite 1 in h.p.l.c. system a₉ and to metabolite 2 in h.p.l.c. system a₆ were collected and evaporated down to dryness.

The mass spectra for both metabolites have a molecular ion peak at m/e 209, but are distinguishable from relative peak intensities (see Figure 3.12). The spectrum for metabolite 1 is identical to that of N-methyl-7,8-benzo-2-quinolone and also resembles the spectrum for N-methyl-2-quinolone in that the most intense peak after the molecular ion is at M-29 (M-HCO). The spectrum for metabolite 2 was found to resemble that for N-methyl-4-quinolone as both spectra show an intense peak at M-28 (M-CO). The mass spectral data is consistent with the incubation products being isomeric.

The infrared spectra of metabolite 1 and N-methyl-7,8-benzo-2-quinolone are identical, both exhibiting a strong carbonyl stretching band at 1650 cm⁻¹ (see Figure 3.13). The spectrum of metabolite 2 also has a band in the carbonyl stretching region but at lower frequency, 1630 cm⁻¹ which is characteristic of a 4-quinolone.

The identity of metabolite 1 as N-methyl-7,8-benzo-2-quinolone was further confirmed on comparison of the n.m.r. spectra (see Figure 3.14).
Figure 3.12. Mass spectra of N-methyl-7,8-benzo-2-quinolone and the incubation products of N-methyl-7,8-benzoquinolinum.
Figure 3.13. Infrared spectra of N-methyl-7,8-benzo-2-quinolone and the incubation products of N-methyl-7,8-benzoquinolinium.
Figure 3.14. N.m.r. spectra of N-methyl-7,8-benzo-2-quinolone and one of the incubation products of N-methyl-7,8-benzoquinolinium

N-Methyl-7,8-benzo-2-quinolone

Metabolite 1

* CHCl₃ impurity in CDC1₃
Metabolite 1 was isolated to obtain this spectrum using preparative t.l.c. with solvent F as the mobile phase. Unfortunately insufficient sample prevented a n.m.r. spectrum of metabolite 2 being obtained.

Thus metabolite 1 has been established as being N-methyl-7,8-benzo-2-quinolone and although unable to compare metabolite 2 with an authentic sample of N-methyl-7,8-benzo-4-quinolone, from the spectral evidence presented, it would in fact appear to be the 4-quinolone.

A greater proportion of the 4-quinolone was produced on incubation with guinea pig enzyme than with rabbit enzyme as shown in Figure 3.15

Figure 3.15. H.p.l.c. traces of the incubation products of N-methyl-7,8-benzoquinolinium perchlorate*

(i) with rabbit enzyme  (ii) with guinea pig enzyme

* H.p.l.c. system a₅
3.6 Oxidation Product of N-Methyl-5,6-benzoquinolinium Salts

N-Methyl-5,6-Benzoquinolinium

Only one product was detected on incubation of N-methyl-5,6-benzoquinolinium with aldehyde oxidase from either rabbit or guinea pig.

*T.l.c. data*

<table>
<thead>
<tr>
<th></th>
<th>Solvent A</th>
<th>Solvent C</th>
<th>Solvent H</th>
<th>Detection Tests</th>
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<td>benzoquinolinium+</td>
<td>s</td>
<td>0</td>
<td>0</td>
<td>purple +</td>
</tr>
<tr>
<td>X</td>
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</tr>
<tr>
<td>Metabolite</td>
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<td>40</td>
<td>16</td>
<td>purple +</td>
</tr>
<tr>
<td>N-Methyl-5,6-benzo-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-quinolone</td>
<td>75</td>
<td>40</td>
<td>16</td>
<td>purple +</td>
</tr>
</tbody>
</table>

Conditions as in section 2.5.1. X = Cl, ClO₄⁻, I
H.p.l.c. data

<table>
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<th>Retention Volumes/ml</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>a₆</td>
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<td>n</td>
</tr>
<tr>
<td>Metabolite</td>
<td>6</td>
</tr>
<tr>
<td>N-Methyl-5,6-benzo-2-quinolone</td>
<td>6</td>
</tr>
</tbody>
</table>

Conditions as in section 2.5.2

The infrared spectrum for the metabolite is characterised by a carbonyl band at 1650 cm⁻¹ and is identical to that of N-methyl-5,6-benzo-2-quinolone (see Figure 3.16). As shown in Figure 3.17 the mass spectra were also found to be identical with a molecular ion peak at m/e 209 and the most abundant fragment ion peak at m/e 181 (M-CO).

Thus it would appear that aldehyde oxidase catalysed oxidation of N-methyl-5,6-benzoquinolinium yields N-methyl-5,6-benzo-2-quinolone. From the results presented thus far, and in particular by analogy with the isomeric N-methyl-7,8-benzoquinolinium, one may have expected two incubation products - the other one being N-methyl-5,6-benzo-4-quinolone. No reports on the synthesis of this compound have appeared in the literature. Furthermore preparation by N-methylation of 4-hydroxy-5,6-benzoquinoline was not attempted because this compound is prepared from β-naphthylamine, an extremely potent carcinogen, the sale of which has been banned. So without a sample of the authentic compound it is difficult to entirely eliminate the possibility of oxidation also occurring at the carbon γ to the ring nitrogen. (Note: On one or two occasions using guinea pig enzyme a faint spot was observed by t.i.c. with Rf values in the region one may have expected for the 4-quinolone, so it is possible that this compound is produced in trace amounts).
Figure 3.16. Infrared spectra of 8-methyl-5,6-benzo-2-quinolone and the incubation product of 8-methyl-5,6-benzoquinolinium
Figure 3.17. Mass spectra of N-methyl-5,6-benzo-2-quinolone and the incubation product of N-methyl-5,6-benzoquinolinium

N-Methyl-5,6-benzo-2-quinolone

Metabolite
3.7 Oxidation Product of N-Methylphenanthridinium Salts

T.L.c. analysis of dichloromethane extracts resulting from the incubation of N-methylphenanthridinium salts with either rabbit or guinea pig enzyme indicated formation of one incubation product. Isolation of this metabolite was relatively easy as it was found to precipitate out of the incubation systems and the solid so formed was filtered off and from t.L.c. found to be free of contaminating substrate or enzyme.

_Chromatographic data_

<table>
<thead>
<tr>
<th></th>
<th>T.L.c. data</th>
<th>H.p.l.c. data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$R_f \times 100$</td>
<td>Retention Volumes/ml</td>
</tr>
<tr>
<td>Solvent A</td>
<td>Solvent B</td>
<td>$a_{10}$</td>
</tr>
<tr>
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<td>s</td>
<td>0</td>
</tr>
<tr>
<td>Metabolite</td>
<td>77.5</td>
<td>60</td>
</tr>
<tr>
<td>N-Methylphenanthridone</td>
<td>77.5</td>
<td>60</td>
</tr>
</tbody>
</table>

* Spots detected at 253.7 nm and 375 nm. H.p.l.c. conditions as described in section 2.5.2 except monitored at 254 nm.

Infrared, n.m.r. and mass spectra were measured of the solid isolated from incubation mixtures and compared with spectra obtained from N-methylphenanthridone as shown in Figures 3.18, 3.19 and 3.20.

The infrared spectrum of the metabolite is very clearly resolved with a strong carbonyl band at 1655 cm$^{-1}$ and corresponds to that for N-methylphenanthridone (Figure 3.18). The mass spectra were very similar with a molecular ion peak at m/e 209 and the most abundant fragment ion at m/e 178 (Figure 3.19).

The identity of the metabolite is unequivocally established as
Figure 3.18. Infrared spectra of N-methylphenanthridone and the incubation product of N-methylphenanthridinium
Figure 3.19. Mass spectra of N-methylphenanthridone and the incubation product of N-methylphenanthridinium.
Figure 3.20. N.m.r. spectra of N-methylphenanthridone and the incubation product of N-methylphenanthridinium

N-Methylphenanthridone

Metabolite

* CHCl₃ impurity in CDCl₃
N-methylphenanthridone on comparison of the n.m.r. spectra which are identical (Figure 3.20), both having the following resonances 63.81 (s, N-CH$_3$), 6.46-7.84 (m, aromatic H), 8.26 (d, 1H J 7.33 Hz), and 8.55 (m, 1H). Therefore aldehyde oxidase catalysed oxidation of N-methylphenanthridinium occurs α to the ring nitrogen.

3.8 Oxidation Product of N-Methyl-4-methylquinolinium Perchlorate

One product was formed on incubation of N-methyl-4-methylquinolinium perchlorate with partially purified aldehyde oxidase from either rabbit or guinea pig.

**Chromatographic data**

<table>
<thead>
<tr>
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<th>H.p.l.c. data</th>
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<td>a$_8$</td>
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<td>Solvent B</td>
<td>Solvent C</td>
<td>Solvent E</td>
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<td>N-Methyl-4-methylquinolinium$^+$ ClO$_4^-$</td>
<td>s</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Metabolite</td>
<td>60</td>
<td>29</td>
<td>34</td>
</tr>
<tr>
<td>N-Methyl-4-methyl-2-quinolone</td>
<td>60</td>
<td>29</td>
<td>34</td>
</tr>
</tbody>
</table>

Conditions as described in sections 2.5.1 and 2.5.2.

* Spots detected at 253.7 nm and 375 nm.

The u.v. spectrum of the metabolite is typical of a 2-quinolone (i.e. strongly absorbing at ca. 270 nm and exhibiting no shift in acid).
Figure 3.21. Mass spectra of N-methyl-4-methyl-2-quinolone and the incubation product of N-methyl-4-methylquinolinium
Figure 3.22. Infrared spectra of N-methyl-4-methyl-2-quinolone and the incubation product of N-methyl-4-methylquinolinium.
### U.v. spectral data

<table>
<thead>
<tr>
<th></th>
<th>$\lambda_{\text{max}}$/nm</th>
<th>(i)</th>
<th>(ii)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Methyl-4-methylquinolinium$^+$ ClO$_4^-$</td>
<td>314</td>
<td>no change</td>
<td></td>
</tr>
<tr>
<td>Metabolite</td>
<td>324,272</td>
<td>no change</td>
<td></td>
</tr>
<tr>
<td>N-Methyl-4-methyl-2-quinolone</td>
<td>324,272</td>
<td>no change</td>
<td></td>
</tr>
</tbody>
</table>

(i) measured in Sørensens phosphate buffer pH 7.
(ii) spectra after adjustment to pH 1 with HCl.

The mass spectrum for the metabolite is identical to that obtained for N-methyl-4-methyl-2-quinolone (see Figure 3.21), with a molecular ion peak at m/e 173 and the most abundant fragment ion peak at m/e 144 (M-HCO). The similarity of the infrared spectra (characterised by a carbonyl band at 1655 cm$^{-1}$, see Figure 3.22) further confirmed the identity of the aldehyde oxidase catalysed oxidation product as N-methyl-4-methyl-2-quinolone.

### 3.9 Oxidation Product of N-Methyl-2-methylquinolinium Salts

In 1960 the oxidation of N-ethyl-2-methylquinolinium iodide with a rabbit liver enzyme preparation was investigated by Otaka et al.$^{211}$ The results are outlined below:
(i) Anaerobic conditions (using methylene blue as electron acceptor)

![Chemical structure]

(ii) Aerobic conditions

![Chemical structure]

Major metabolite

Exhaustive chromatographic and extraction procedures were used to isolate the products which were identified by u.v. and t.l.c. Hydrogen peroxide was produced during aerobic conversion of (A) to (B) and was suggested to chemically oxidise (A) to the postulated intermediate (a). The enzyme responsible for oxidation of (a) to (C) was thought to be the same enzyme as was studied by Knox \(^{21}\) (i.e. aldehyde oxidase). However, because Knox found quinoline compounds to be oxidised by aldehyde oxidase at the carbon atom adjacent to the ring nitrogen, Otaka \textit{et al.} assumed that oxidation of (A) to (B) i.e. at a carbon removed from nitrogen, must be due to a different enzyme present in the preparation. In light of the results presented thus far in this thesis, one would now expect aldehyde oxidase to be capable of oxidation at carbon 4.
Only one metabolite was detected on aerobic incubation of N-methyl-2-methylquinolinium salts with aldehyde oxidase from either rabbit or guinea pig. In view of the results previously reported, two metabolites were anticipated, N-methyl-2-methyl-4-quinolone and N-methyl-2-methyl-4-quinolone-2-carboxylic acid.

Dichloromethane extracts were used to obtain t.l.c. and adsorption system h.p.l.c. \( (a_3) \) data for the metabolite. In addition further incubations were carried out which were terminated by the addition of methanol and aliquots of the aqueous methanolic solutions were then injected directly on to the reverse phase h.p.l.c. columns \( (p_3, p_4) \). This method eliminates the problems associated with extraction (particularly if one was considering extraction of an acid).

**T.L.C. data**

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Rf x 100</th>
<th>Detection Tests</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(i) 375 nm (ii) 253.7 nm</td>
</tr>
<tr>
<td>A</td>
<td>Solvent B</td>
<td>Solvent G</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Solvent A</th>
<th>Solvent B</th>
<th>Solvent G</th>
<th>(i) 375 nm</th>
<th>(ii) 253.7 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Methyl-2-methylquinolinium+X^-</td>
<td>s</td>
<td>s</td>
<td>10</td>
<td>purple</td>
<td>+</td>
</tr>
<tr>
<td>Metabolite</td>
<td>75</td>
<td>42</td>
<td>55</td>
<td>yellow</td>
<td>+</td>
</tr>
<tr>
<td>N-Methyl-2-methyl-4-quinolone</td>
<td>75</td>
<td>42</td>
<td>55</td>
<td>yellow</td>
<td>+</td>
</tr>
<tr>
<td>N-Methyl-4-quinolone-2-carboxylic acid</td>
<td>28</td>
<td>s</td>
<td>68</td>
<td>purple</td>
<td>+</td>
</tr>
</tbody>
</table>

Conditions as described in section 2.5.1. \( X = I, ClO_4^- \)
### H.p.l.c. data

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>a5</th>
<th>p3</th>
<th>p4</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Methyl-2-methylquinolinium X^-</td>
<td>13.2</td>
<td>n</td>
<td>3.5</td>
</tr>
<tr>
<td>Metabolite</td>
<td>8</td>
<td>n</td>
<td>7</td>
</tr>
<tr>
<td>N-Methyl-2-methyl-4-quinolone</td>
<td>8</td>
<td>n</td>
<td>7</td>
</tr>
<tr>
<td>N-Methyl-4-quinolone-2-carboxylic acid</td>
<td>n</td>
<td>10.2</td>
<td>4.5</td>
</tr>
</tbody>
</table>

Conditions as in section 2.5.2, except monitored at 320 nm.

A sample of the metabolite was isolated using adsorption h.p.l.c. system a5 and subjected to infrared and mass spectral analysis.

The infrared spectrum of the metabolite as shown in Figure 3.23 is characterised by a peak in the carbonyl stretching region at 1625 cm\(^{-1}\) and corresponds to that for N-methyl-2-methyl-4-quinolone. In contrast, the spectrum of the acid has two carbonyl stretching frequencies at 1660 and 1630 cm\(^{-1}\).

The mass spectra of the metabolite and N-methyl-2-methyl-4-quinolone also correspond, both exhibiting the molecular ion peak, M, at m/e 173 (Figure 3.24). The mass spectrum of the acid on the other hand has a molecular ion peak at m/e 203 and a different fragmentation pattern.

The identity of the metabolite was further confirmed by u.v. spectroscopy.
Figure 3.23. Infrared spectra of N-methyl-2-methyl-4-quinolone and the incubation product of N-methyl-2-methylquinolinium.
Figure 3.24. Mass spectra of N-methyl-2-methyl-4-quinolone and the incubation product of N-methyl-2-methylquinolinium.
**U.v. spectral data**

<table>
<thead>
<tr>
<th></th>
<th>( \lambda_{\text{max}} / \text{nm} ) (i)</th>
<th>( \lambda_{\text{max}} / \text{nm} ) (ii)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Methyl-2-methylquinolinium(^+) I(^-)</td>
<td>320</td>
<td>no change</td>
</tr>
<tr>
<td>Metabolite</td>
<td>334,330,321</td>
<td>306</td>
</tr>
<tr>
<td>N-Methyl-2-methyl-4-quinolone</td>
<td>334,330,320</td>
<td>306</td>
</tr>
<tr>
<td>N-Methyl-2-methyl-4-quinolone-2-carboxylic acid</td>
<td>289</td>
<td>332(sh),320</td>
</tr>
</tbody>
</table>

sh = shoulder. (i) measured in Sørensen's phosphate buffer pH 7. (ii) spectra after adjustment to pH 1 with HCl.

The n.m.r. spectrum of the metabolite, which was isolated using preparative t.l.c. with solvent B as the mobile phase, was characterised by the following resonances: \( \delta \) 2.48 (s, C\(_2\)-CH\(_3\)), 3.73 (s, N-CH\(_3\)), 6.22 (s, C\(_3\)-H) and 8.45 (m, C\(_5\)-H) which corresponded to those for N-methyl-2-methyl-4-quinolone. However, the spectrum showed that the isolated enzymatic oxidation product was contaminated with substrate.

Thus it would appear that aldehyde oxidase catalysed oxidation of N-methyl-2-methylquinolinium yields only one oxidation product which was identified as N-methyl-2-methyl-4-quinolone; no N-methyl-2-methyl-4-quinolone-2-carboxylic acid was detected. Oxidation of this cation is therefore apparently different from that reported for the N-ethyl analogue;\(^{211}\) this may be because:

(i) The N-ethyl compound interacts differently with the enzyme.

(ii) The investigation with N-ethyl-2-methylquinolinium was carried out using a much cruder preparation of the enzyme.

(iii) The acid is derived from chemical oxidation occurring during a lengthy extraction procedure.

Explanation (ii) can be eliminated since incubation of N-methyl-2-methyl-
quinolinium with fresh liver slices gave the same results that had been obtained with partially purified aldehyde oxidase. Incubations with the latter enzyme preparation in the presence of catalase (1 mg/ml) made no significant difference to the amount of N-methyl-2-methyl-4-quinolone isolated and hence hydrogen peroxide is not involved in this oxidation. To determine if (i) or (iii) were valid it would be necessary to repeat the work of Otaka et al. Further investigations were carried out on quinolinium compounds substituted in the 2 position but with the potentially labile 2-methyl substituent being replaced by a phenyl group (see sections 3.10 and 3.12).

Note concerning the following sections (3.10, 3.11 and 3.12)

Metabolites of N-methyl-2-phenylquinolinium, N-methyl-4-phenylquinolinium and N-phenyl-2-phenylquinolinium, were isolated and identified from incubations with aldehyde oxidase derived from rabbit rather than from guinea pig. This was because although reasonable rates of oxidation were monitored spectrophotometrically for these compounds with rabbit enzyme, only very poor rates were observed using guinea pig enzyme (for method see section 2.6.2.1). Furthermore incubation of N-methyl-2-phenylquinolinium with the latter enzyme yielded only ca. 5% of the product obtained with rabbit enzyme. Thus it would appear that a phenyl group in position 2 or 4 hinders the catalytic ability of guinea pig enzyme but not of rabbit enzyme; this interesting finding is further discussed in Chapter 4.

3.10 Oxidation Product of N-Methyl-2-phenylquinolinium Perchlorate

T.l.c. and h.p.l.c. analysis of dichloromethane extracts resulting from the incubation of N-methyl-2-phenylquinolinium perchlorate with
partially purified aldehyde oxidase from rabbit, indicated formation of one metabolite.

Chromatographic data

<table>
<thead>
<tr>
<th>T.l.c. data*</th>
<th>H.p.l.c. data</th>
</tr>
</thead>
<tbody>
<tr>
<td>( R_f \times 100 )</td>
<td>Retention Volumes/ml</td>
</tr>
<tr>
<td>Solvent A</td>
<td>Solvent D</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>N-Methyl-2-phenyl-quinolinium(^+) (\text{C}1\text{O}_4^-)</td>
<td>s</td>
</tr>
<tr>
<td>Metabolite</td>
<td>72</td>
</tr>
</tbody>
</table>

Conditions as in sections 2.5.1 and 2.5.2. * Spots detected at 253.7 nm and 375 nm.

Samples of the metabolite were collected from the h.p.l.c. eluate (system a\(_3\)) and the u.v., infrared and mass spectra were measured.

The u.v. spectrum for the metabolite showed a bathochromic shift in acid which is characteristic of 4-quinolones and the \(\lambda_{\text{max}}\) values correspond to those reported for N-methyl-2-phenyl-4-quinolone.

U.v. spectral data

<table>
<thead>
<tr>
<th>(\lambda_{\text{max}}/\text{nm} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i)</td>
</tr>
<tr>
<td>N-Methyl-2-phenyl-quinolinium(^+) (\text{C}1\text{O}_4^-)</td>
</tr>
<tr>
<td>Metabolite</td>
</tr>
<tr>
<td>N-Methyl-2-phenyl-4-quinolone</td>
</tr>
</tbody>
</table>

1. measured in Sørensen's phosphate buffer pH 7. 2. spectra after adjustment to pH 1 with HCl. 3. recorded by Johnstone et al. in ethanol\(^2\). 4. recorded by Goodwin et al. in 0.9 M HCl\(^2\).
Figure 3.25. Infrared spectra of N-methyl-2-phenyl-4-quinolone and the incubation product of N-methyl-2-phenylquinolinium

Figure 3.26. Mass spectrum of the incubation product of N-methyl-2-phenylquinolinium
In Figure 3.25 the infrared spectra of the metabolite (KBr micro-disc) and N-methyl-2-phenyl-4-quinolone (measured in nujol by Price and Willis214) are compared and found to be very similar with both exhibiting a carbonyl stretching band at 1620 cm\(^{-1}\). The mass spectral data also corresponded (data for N-methyl-2-phenyl-4-quinolone recorded by Tillequin and Sevenet215) both spectra have a molecular ion peak at m/e 235 and the most abundant fragment ion peak at m/e 207 (M-CO). Figure 3.26 shows the mass spectrum of the metabolite.

Thus aldehyde oxidase catalysed oxidation of N-methyl-2-phenyl-quinolininium occurs at the carbon \(\gamma\) to the ring nitrogen.

3.11 Oxidation Product of N-Methyl-4-phenylquinolinium Salts

One product was formed on incubation of N-methyl-4-phenylquinolinium with rabbit liver aldehyde oxidase.

**T.l.c. data**

<table>
<thead>
<tr>
<th>Solvent</th>
<th>R(_f) x 100</th>
<th>Detection Tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Solvent C</td>
<td>(i) 375 nm (ii) 253.7 nm</td>
</tr>
<tr>
<td>Solvent E</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Methyl-4-phenyl-quinolinium(^+) X(^-)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Metabolite</td>
<td>64</td>
<td>51</td>
</tr>
<tr>
<td>N-Methyl-4-phenyl-2-quinolone</td>
<td>64</td>
<td>51</td>
</tr>
</tbody>
</table>

Conditions as in section 2.5.1. \(X = \text{I, ClO}_4\). \(*\) faint spot
**H.p.l.c. data**

<table>
<thead>
<tr>
<th></th>
<th>Retention Volumes/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( a_7 ) ( a_9 )</td>
</tr>
<tr>
<td>N-Methyl-4-phenylquinolinium ( + X^- )</td>
<td>n         ( a_9 )</td>
</tr>
<tr>
<td>Metabolite</td>
<td>8.5       11.7</td>
</tr>
<tr>
<td>N-Methyl-4-phenyl-2-quinolone</td>
<td>8.5       11.7</td>
</tr>
</tbody>
</table>

Conditions as in section 2.5.2

Eluates corresponding to the metabolite in incubation system \( a_7 \) were collected and evaporated to dryness. The residue so obtained was subjected to infrared, u.v., n.m.r. and mass spectral analysis.

As shown in Figure 3.27 the infrared spectra of the metabolite is characterised by a carbonyl stretching band at 1660 cm\(^{-1}\) and is identical to that for N-methyl-4-phenyl-2-quinolone. The mass spectra were also found to correspond (see Figure 3.28) both having a molecular ion peak at m/e 235 and the most abundant fragment ion peak at 207 (M-CO).

The identity of the metabolite was further confirmed from the u.v. spectra.

**U.v. spectral data**

<table>
<thead>
<tr>
<th></th>
<th>( \lambda_{max}/nm )</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i)</td>
<td>(ii)</td>
</tr>
<tr>
<td>N-Methyl-4-phenylquinolinium ( + I^- )</td>
<td>320                no change</td>
</tr>
<tr>
<td>Metabolite</td>
<td>330,282                no change</td>
</tr>
<tr>
<td>N-Methyl-4-phenyl-2-quinolone</td>
<td>330,282                no change</td>
</tr>
</tbody>
</table>

(i) measured in Sørensen's phosphate buffer pH 7. (ii) spectra after adjustment to pH 1 with HCl.
Figure 3.27. Infrared spectra of N-methyl-4-phenyl-2-quinolone and the incubation product of N-methyl-4-phenylquinolintum.
Figure 3.28. Mass spectra of \(N\)-methyl-4-phenyl-2-quinolone and the incubation product of \(N\)-methyl-4-phenylquinolinium
Figure 3.29. N.m.r. spectra of N-methyl-4-phenyl-2-quinolone and the incubation product of N-methyl-4-phenylquinolinium

N-Methyl-4-phenyl-2-quinolone

Metabolite

* CHCl₃ impurity in CDCl₃
The metabolite was isolated from large scale incubations using preparative t.l.c. with solvent C as the mobile phase and its n.m.r. spectrum recorded. Apart from the splitting pattern of the aromatic region, this spectrum was characterised by a singlet resonance at $\delta 6.69$ due to the C$_3$ proton and the resonance due to N-CH$_3$ at 3.79; furthermore it was found to be identical to that for N-methyl-4-phenyl-2-quinolone (see Figure 3.29).

Thus the product resulting from rabbit liver aldehyde oxidase catalysed oxidation of N-methyl-4-phenylquinolinium was identified as N-methyl-4-phenyl-2-quinolone.

### 3.12 Oxidation Product of N-Phenyl-2-phenylquinolinium Perchlorate

T.l.c. analysis of dichloromethane extracts resulting from the incubation of N-phenyl-2-phenylquinolinium perchlorate with rabbit liver aldehyde oxidase indicated formation of one product. Isolation of this product was relatively easy as it precipitated out of the incubation systems and the solid so formed, was filtered off and from t.l.c. found to be free of contaminating substrate or enzyme.

**T.l.c. data**

<table>
<thead>
<tr>
<th>Solvent C</th>
<th>Solvent D</th>
<th>Solvent H</th>
<th>Detection Tests (i) 375 nm</th>
<th>(ii) 253.7 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>R$_f$ x 100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Phenyl-2-phenyl-quinolinium$^+$ ClO$_4^-$</td>
<td>0</td>
<td>s</td>
<td>s</td>
<td>purple</td>
</tr>
<tr>
<td>Metabolite</td>
<td>46</td>
<td>84</td>
<td>58</td>
<td>-</td>
</tr>
</tbody>
</table>

Conditions as outlined in section 2.5.1
The u.v. spectrum of the metabolite showed splitting of the near ultra-violet maximum into two peaks at pH 7 and a bathochromic shift in acid; both these features are characteristic of 4-quinolones.

**U.v. spectral data**

<table>
<thead>
<tr>
<th></th>
<th>(\lambda_{\text{max}}/\text{nm})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i)</td>
<td>(ii)</td>
</tr>
<tr>
<td>N-Phenyl-2-phenylquinolinium+ C(_{104})⁻</td>
<td>335</td>
</tr>
<tr>
<td>Metabolite</td>
<td>336,324</td>
</tr>
</tbody>
</table>

(i) measured in Sørensen's phosphate buffer pH 7. (ii) spectra after adjustment to pH 1 with HCl.

The infrared spectrum of the solid is clearly resolved (see Figure 3.30) and has a carbonyl stretching band at \(1630\ \text{cm}^{-1}\) and another intense band at \(1600\ \text{cm}^{-1}\), both of which are characteristic of 4-quinolones. The mass spectrum has a molecular ion peak at 298 and the fragmentation pattern indicates initial loss of CO (see Figure 3.31). This data is consistent with the metabolite being N-phenyl-2-phenyl-4-quinolone which is confirmed by its n.m.r. spectrum. The singlet resonance at \(66.44\) is due to the proton on \(\text{C}_3\) and the low field resonance \(68.53\) (m) is assigned to the proton on \(\text{C}_5\) which is deshielded as a result of peri interaction with the \(\text{C}_4\) carbonyl (Figure 3.32).

Thus N-phenyl-2-phenylquinolinium is oxidised by aldehyde oxidase at a carbon removed from the ring nitrogen, to yield N-phenyl-2-phenyl-4-quinolone which was unequivocally identified from the spectroscopic data.
Figure 3.30. Infrared spectrum of the incubation product of N-phenyl-2-phenyquinolinium

Figure 3.31. Mass spectrum of the incubation product of N-phenyl-2-phenyquinolinium
Figure 3.32. N.m.r. spectrum of the incubation product of N-phenyl-2-phenylquinolinium

* CHCl₃ impurity in CDCl₃
3.13 Discussion

Products resulting from incubation of various quinolinium cations with partially purified aldehyde oxidase were isolated and identified. The compounds investigated, which all had a basic quinolinium ring structure with either a methyl or phenyl group on nitrogen, can be divided into three categories:

I. Not substituted α or γ to the ring nitrogen, e.g. N-phenyl-quinolinium.

II. Substituted γ to the ring nitrogen, e.g. N-methyl-4-phenyl-quinolinium.

III. Substituted α to the ring nitrogen, e.g. N-phenyl-2-phenylquinolinium.

Each of these groups will be discussed in turn.

I. Incubation of N-methylquinolinium with aldehyde oxidase yielded two products which were identified as N-methyl-2-quinolone and N-methyl-4-quinolone. Previously this cation had been reported to give only one oxidation product, i.e. the 2-quinolone.²,³ Formation of the 4-quinolone was probably overlooked for the following reasons:

(a) It is a minor product, e.g. with rabbit enzyme (the usual source of enzyme used in these studies) it contributes only ca. 5% of the total product.

(b) Aldehyde oxidase catalysed oxidation is usually considered to occur at a carbon adjacent to nitrogen; thus production of N-methyl-4-quinolone was not expected. Furthermore the formation of two products simultaneously from one substrate is also unusual.

(c) The product was identified by u.v. spectroscopy:³ the spectrum of the 4-quinolone would have been obscured by that of the major product.

N-phenylquinolinium similarly yielded two incubation products, N-phenyl-2-quinolone and N-phenyl-4-quinolone. With both N-methyl and
N-phenylquinolinium cations, the ratio of 2- to 4-quinolones formed depended on the source of aldehyde oxidase used, as shown below.

![Chemical Structure]

<table>
<thead>
<tr>
<th></th>
<th>Rabbit Enzyme</th>
<th>Guinea Pig Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>R = methyl</strong></td>
<td>19 ± 2</td>
<td>10 ± 1</td>
</tr>
<tr>
<td><strong>R = phenyl</strong></td>
<td>4.7 ± 0.4</td>
<td>0.077 ± 0.0085</td>
</tr>
</tbody>
</table>

The following conclusions can be drawn from these results:

(a) Guinea pig aldehyde oxidase gives a higher proportion of the 4-quinolone with both substrates.

(i) With either source of enzyme the 4-quinolone from N-methylquinolinium remained the minor product.

(ii) The source of enzyme used had a dramatic effect on the products obtained from N-phenylquinolinium: the major metabolite was found to be N-phenyl-2-quinolone with rabbit enzyme but with guinea pig enzyme was N-phenyl-4-quinolone.

(b) Replacing the methyl group at nitrogen by a bulkier phenyl substituent resulted in a greater proportion of 4-quinolone being formed regardless of the source of enzyme used.
From preliminary studies using baboon liver aldehyde oxidase the major product resulting from incubation of N-phenylquinolinium appeared to be N-phenyl-4-quinolone.

The 2- and 4-quinolones are genuine products of aldehyde oxidase catalysed oxidation. Experiments were carried out which eliminated the possibility of other enzymatic or non-enzymatic pathways which might have accounted for the hitherto unexpected simultaneous production of the 4-quinolones along with the 2-isomers; these included further purification of the enzyme and the use of a potent inhibitor of aldehyde oxidase, menadione. Moreover the oxygen incorporated into both products was found to be derived from water not oxygen (see section 3.4).

Incubation of N-methyl-7,8-benzoquinolinium salts with partially purified aldehyde oxidase gave two products, N-methyl-7,8-benzo-2-quinolone and N-methyl-7,8-benzo-4-quinolone. The proportion of the 4-quinolone formed was, as expected from the above results, greater with guinea pig enzyme than with rabbit enzyme. In contrast, only one product was isolated on incubation of N-methyl-5,6-benzoquinolinium which was identified as N-methyl-5,6-benzo-2-quinolone. However, as noted in section 3.6, trace amounts of another product were detected by t.l.c. and this may possibly be the 4-quinolone. The reduced ability of aldehyde oxidase to catalyse the oxidation of the carbon Y to the ring nitrogen of N-methyl-5,6-benzoquinolinium compared with the isomer N-methyl-7,8-benzoquinolinium may be a result of steric hindrance caused by the 5,6-benzene ring of the former cation.
Prior to this present study, only one substrate i.e. N¹-methyl-nicotinamide had been found to give two products simultaneously with aldehyde oxidase (see section 1.2.2).

Quinolinium compounds are known to be susceptible to nucleophilic attack at positions 2 and 4.²¹⁶,²¹⁷ It seems generally accepted that strong nucleophiles attack the 2-position whereas weak nucleophiles attack the 4-position,²¹⁸,²¹⁹ though the evidence in favour of this view is not unequivocable.²²⁰,²²¹ It is unlikely that chemical considerations of the positions of nucleophilic attack alone, account for the ratio of 2- to 4-quinolone formed as a result of enzymatic oxidation since the ratios depend on the source of enzyme used.

Since N-methyl and N-phenyl quinolinium cations were found to be oxidised at C-2 and C-4 with aldehyde oxidase, it was decided to see what effect blocking one or other of these positions would have on the course of oxidation. This would be particularly interesting with C-2 substituted cations where the 4-quinolone may have been produced exclusively.

II. Aldehyde oxidase catalysed oxidation of the substituted cations shown below occurred at the carbon atom α to the ring nitrogen.

\[
\begin{align*}
\text{N-Methyl-4-methylquinolinium} & \quad \text{N-Methylphenanthridinium} \\
\text{CH}_3 & \quad \text{CH}_3 \\
\text{CH}_3 & \quad \text{CH}_3
\end{align*}
\]
Prior to this present study, only one substrate \textit{i.e.} \(N^1\)-methyl-nicotinamide had been found to give two products simultaneously with aldehyde oxidase (see section 1.2.2).

Quinolinium compounds are known to be susceptible to nucleophilic attack at positions 2 and 4\(^{216,217}\). It seems generally accepted that strong nucleophiles attack the 2-position whereas weak nucleophiles attack the 4-position\(^{218,219}\), though the evidence in favour of this view is not unequivocal\(^{220,221}\). It is unlikely that chemical considerations of the positions of nucleophilic attack alone, account for the ratio of 2- to 4-quinolone formed as a result of enzymatic oxidation since the ratios depend on the source of enzyme used.

Since \(N\)-methyl and \(N\)-phenyl quinolinium cations were found to be oxidised at C-2 and C-4 with aldehyde oxidase, it was decided to see what effect blocking one or other of these positions would have on the course of oxidation. This would be particularly interesting with C-2 substituted cations where the 4-quinolone may have been produced exclusively.

II. Aldehyde oxidase catalysed oxidation of the substituted cations shown below occurred at the carbon atom \(\alpha\) to the ring nitrogen.

\[
\begin{align*}
\text{N-Methyl-4-methylquinolinum} & & \text{N-Methylphenanthridinium} \\
\text{N-Methyl-4-phenylquinolinium}
\end{align*}
\]
III. Incubation of the cations shown below with partially purified aldehyde oxidase yielded the corresponding 4-quinolone.

![Chemical structures](image)

N-Methyl-2-methylquinolinium

N-Methyl-2-phenylquinolinium

N-Phenyl-2-phenylquinolinium

It has been shown in this chapter that quinolinium cations substituted at C-2 are oxidised at the carbon γ to the ring nitrogen, thus questioning the previously held view that it is *exceptional* for aldehyde oxidase to oxidise a carbon other than that adjacent to a ring nitrogen.
CHAPTER 4

Kinetic Studies of Quinolinium and Related Cations

With Aldehyde Oxidase

4.1 Introduction

In the last chapter it was shown that aldehyde oxidase is capable of catalysing the oxidation of quinolinium compounds not only at the carbon α, but also the carbon γ to the ring nitrogen. In addition, preliminary studies revealed that with compounds having a phenyl group substituted at either carbon 2 or 4, the turnover rate with guinea pig enzyme is considerably lower than with rabbit enzyme. In view of these interesting findings it was decided to carry out a more detailed kinetic study.

4.2 Determination of $K_m$ and $K_i$ Values

Spectrophotometric assays were developed to determine Michaelis-Menten constants ($K_m$) (see experimental sections 2.6.2.1, 2.6.2.2 and 2.6.2.3). The method used, unless otherwise stated employed potassium ferricyanide as the electron acceptor and reaction rates were monitored by observing the change in absorbance that occurs at 420 nm, as the ferricyanide was reduced to ferrocyanide. The iodide salts of the N-heterocyclic cations were not used in these kinetic studies as iodide ions interfere with ferricyanide.\(^3^4\) For the sake of direct comparison, where possible, the perchlorate salts of each N-heterocycle were studied.

Partially purified enzyme was used (preparation, see section 2.2.1) and the rates, which were usually constant for the first two to five minutes, were measured for various concentrations of substrate. The concentrated enzyme preparation was diluted as necessary to give suitable reaction rates over the substrate concentration used to obtain the $K_m$. The results were plotted as follows:
(a) $v$ versus $s$, and
(b) $1/v$ versus $1/s$.

If no irregularities were noted from the graphical representations then the $v$ and $s$ values were fed into a computer programme which calculated $K_m$ for the substrate using the Lineweaver-Burk transformation of the Michaelis-Menten equation and also gave the correlation coefficient of the straight line by linear regression.

Some compounds were found to be either only very slowly oxidised or not oxidised at all with the enzyme. In these circumstances the compounds were tested for inhibitory properties by determining if they were able to reduce oxidation rates of other substrates. Inhibitor constants ($K_i$) were measured against 6-methylpurine; this substrate was chosen since it gives good rates and has a reasonable $K_m$ with both guinea pig and rabbit enzyme. Different concentrations of 6-methylpurine and inhibitor were incubated together and the rates were followed at 420 nm using ferricyanide. From the results, a $1/v$ versus $1/s$ graph was plotted for 6-methylpurine in the absence, and presence, of inhibitor and $K_i$ was calculated from this graph.

The $K_m$ and $K_i$ values obtained are given in Table 4.1 and the corresponding graphs are presented below along with comments pertaining to these results. These values are then more fully discussed in section 4.3.
Table 4.1. Michaelis and inhibitor constants for quinolinium and related compounds with rabbit and guinea pig aldehyde oxidase

<table>
<thead>
<tr>
<th>Compounda</th>
<th>Lineweaver-Burk Plotsb</th>
<th>With rabbit enzyme</th>
<th>With guinea pig enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fig./Page</td>
<td>( K_m^b / \text{M} )</td>
<td>( K_i / \text{M} )</td>
</tr>
<tr>
<td>N-Methylquinolinium</td>
<td>4.1a,b; 152</td>
<td>1.6 x 10^{-3} (0.998)</td>
<td>5.0 x 10^{-4} (0.998)</td>
</tr>
<tr>
<td>N-Phenylquinolinium</td>
<td>4.2a,b; 153</td>
<td>7.1 x 10^{-5} (0.999)</td>
<td>7.9 x 10^{-5} (0.998)</td>
</tr>
<tr>
<td>N-Methyl-7,8-benzoquinolinium</td>
<td>4.3a,b; 154</td>
<td>2.3 x 10^{-6} (0.993)</td>
<td>7.1 x 10^{-5} (0.996)</td>
</tr>
<tr>
<td>N-Methyl-5,6-benzoquinolinium</td>
<td>4.4a,b; 155</td>
<td>2.5 x 10^{-6} (0.999)g</td>
<td>7.8 x 10^{-5} (0.994)</td>
</tr>
<tr>
<td>N-Methylphenanthridinium</td>
<td>4.5; 156</td>
<td>1.8 x 10^{-6} (0.999)f</td>
<td>&lt;10^{-5}d</td>
</tr>
<tr>
<td>N-Methyl-4-methylquinolinium</td>
<td>4.6; 156</td>
<td>1.0 x 10^{-3} (0.995)c</td>
<td>g</td>
</tr>
<tr>
<td>N-Methyl-2-methylquinolinium</td>
<td>h</td>
<td>h</td>
<td></td>
</tr>
<tr>
<td>N-Methyl-2-phenylquinolinium</td>
<td>4.7a,b; 157</td>
<td>4.6 x 10^{-6} (0.998)</td>
<td>2.0 x 10^{-5}</td>
</tr>
<tr>
<td>N-Methyl-4-phenylquinolinium</td>
<td>4.8a,b; 158</td>
<td>2.9 x 10^{-6} (0.996)</td>
<td>2.0 x 10^{-5}</td>
</tr>
<tr>
<td>N-Phenyl-2-phenylquinolinium</td>
<td>4.9; 159</td>
<td>&lt;10^{-5}d</td>
<td>8.0 x 10^{-7}</td>
</tr>
<tr>
<td>N-Methylisoquinolinium</td>
<td>4.10a,b;160</td>
<td>2.0 x 10^{-3}</td>
<td>8.0 x 10^{-4}</td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td>4.11a,b;161</td>
<td>4.6 x 10^{-6}</td>
<td>4.6 x 10^{-6}</td>
</tr>
</tbody>
</table>

---

a Unless otherwise stated, anion was perchlorate
b Correlation figures in parentheses
c Oxygen used as electron acceptor, all other figures reported in table use the ferricyanide assay
d In practice the ferricyanide assay cannot be used to obtain accurate values for \( K_m \) below 10^{-5} M, as a result of the small extinction coefficient of ferricyanide
e \( K_m \) also determined using oxygen as the electron acceptor, monitored at 390 nm, 2.6 x 10^{-6} M (0.998)
f \( K_m \) was also determined using oxygen as the electron acceptor, monitored at 365 nm, 1.6 x 10^{-6} M (0.999) - N.B. this assay was not sensitive enough to obtain an accurate value for \( K_m \) <10^{-5} M with guinea pig enzyme
g Not measured
h Found to be neither a good substrate nor a good inhibitor
i Velocities are in arbitrary units calculated from the gradient obtained on the chart paper
Figure 4.1. Determination of the Michaelis constants for N-methylquinolininium

(a) with rabbit aldehyde oxidase

(b) with guinea pig aldehyde oxidase
Figure 4.2. Determination of the Michaelis constants for N-phenylquinolinium

(a) with rabbit aldehyde oxidase

(b) with guinea pig aldehyde oxidase
Figure 4.3. Determination of the Michaelis constants for N-methyl-7,8-benzoquinolinium

(a) with rabbit aldehyde oxidase

(b) with guinea pig aldehyde oxidase

1/s x 10^{-3} / mol^{-1} dm^{3}

1/s x 10^{-3} / mol^{-1} dm^{3}
Figure 4.4. Determination of the Michaelis constants for N-methyl-5,6-benzoquinolinium

(a) with rabbit aldehyde oxidase

(b) with guinea pig aldehyde oxidase
**Figure 4.5.** Determination of the Michaelis constant for N-methylphenanthridinium with rabbit aldehyde oxidase

**Figure 4.6.** Determination of the Michaelis constant for N-methyl-4-methylquinolinium with rabbit aldehyde oxidase
Figure 4.7. *N*-Methyl-2-phenylquinolinium, determination of
(a) its Michaelis constant with rabbit aldehyde oxidase

Oxidation of 6-methylpurine (○) and in the presence of 7.0 × 10⁻⁵ M (△), and
1.2 × 10⁻⁴ M (▲) *N*-methyl-2-phenylquinolinium perchlorate.

(b) its Inhibitor constant with guinea pig aldehyde oxidase
Figure 4.8. N-Methyl-4-phenylquinolinium, determination of
(a) its Michaelis constant with rabbit aldehyde oxidase

Oxidation of 6-methylpurine (●) and in the presence of 1.6 x 10⁻⁵ M (▲),
4.0 x 10⁻⁵ M (▲), and 6.6 x 10⁻⁵ M (○) N-methyl-4-phenylquinolinium
perchlorate.
Figure 4.9. Determination of the Inhibitor constant for N-phenyl-2-phenyl-quinolinium with guinea pig aldehyde oxidase

Oxidation of 6-methylpurine (□) and in the presence of 2.8 x 10⁻⁶ M N-phenyl-2-phenylquinolinium perchlorate (■)
Figure 4.10. Determination of Inhibitor constants for N-methylisoquinolinium

(a) with rabbit aldehyde oxidase

Oxidation of 6-methylpurine (▲) and in the presence of 3.01 x 10^{-3} M (□), and 6.27 x 10^{-3} M (▲) N-methylisoquinolinium perchlorate.

(b) with guinea pig aldehyde oxidase

Oxidation of 6-methylpurine (▲) and in the presence of 9.9 x 10^{-4} M (▼), and 2.0 x 10^{-3} M (○) N-methylisoquinolinium perchlorate.
Figure 4.11. Determination of the Inhibitor constants for ethidium bromide

(a) with rabbit aldehyde oxidase

Oxidation of 6-methylpurine (Δ) and in the presence of 4.9 x 10^{-6} M (▲), 1.0 x 10^{-5} M (□), and 2.0 x 10^{-5} M (■) ethidium bromide

(b) with guinea pig aldehyde oxidase

Oxidation of 6-methylpurine (Δ) and in the presence of 4.8 x 10^{-6} M ethidium bromide (▲)
Comments pertaining to the $K_m$ and $K_i$ determinations

$K_m$ values were obtained for N-methylquinolinium, N-phenylquinolinium, N-methyl-7,8-benzoquinolinium, N-methyl-5,6-benzoquinolinium and N-methyl-phenanthridinium cations with both rabbit and guinea pig enzyme; the $K_m$ values obtained indicated these compounds to be good substrates. It was possible in the case of the latter two compounds to directly observe the decrease in substrate concentration spectrophotometrically. The $K_m$ values measured by this method were found to be in good agreement with those obtained using the ferricyanide assay (see Table 4.1).

The rate of reduction of ferricyanide monitors total product formation. For substrates which yield both 2- and 4-quinolones, it would be difficult to monitor by direct spectrophotometric methods the separate formation of each product since the u.v. spectra for these compounds are too similar. Therefore the oxidation of N-methylquinolinium perchlorate with rabbit enzyme was followed using h.p.l.c. to separate and measure the amounts of each product formed with time as the reaction progressed (see experimental section 2.4.2). While the substrate concentration fell from $1.3 \times 10^{-3}$ M to $4.3 \times 10^{-3}$ M over a period of one hour, the ratio of 2- to 4-quinolone remained constant (i.e. $17 \pm 1$). The rate of formation of either product can be expressed in the form of a progress curve as shown in Figure 4.12.223,224 It can be seen that when the axes are adjusted to take account of the differing amounts of the two products a common curve can be drawn through the data points for each product. This indicates that the same $K_m$ value would be obtained using either product, although it is obvious from the graph that the $V_{max}$ values must be different. Felsted and Chaykin39 found the $K_m$ values for both products of N1-methylnicotinanide to be equal for several sources of aldehyde oxidase, while differences in $V_{max}$ values for the two products accounted for the observed ratios.
Figure 4.12. Progress curve for the production of N-methyl-2-quinolone and N-methyl-4-quinolone from N-methylquinolinium perchlorate

(●) Conc. of 2-quinolone produced $\times 10^4$/mol dm$^{-3}$

(○) Conc. of 4-quinolone produced $\times 10^5$/mol dm$^{-3}$

* H.p.l.c. system p5 used
In the presence of ferricyanide the enzymatic oxidation of both N-methyl-4-methylquinolinium and N-methyl-2-methylquinolinium was found to be inhibited; moreover this effect was found to be peculiar to these two cations and is as yet unexplained. It was noticed that no reduction of ferricyanide was observed for N-methyl-4-methylquinolinium with aldehyde oxidase, but if cytochrome c (experimental section 2.6.2.2) or oxygen replaced ferricyanide as the electron acceptor then reasonable rates were observed. When oxygen was employed as the electron acceptor, reaction rates were monitored by the increase in absorbance at 270 nm due to formation of the 2-quinolone ($\Delta \varepsilon = 5,400$) and the $K_m$ value ($10^{-3}$ M) was determined by this method. Addition of ferricyanide to this assay system however completely inhibited the change in this absorbance. Similarly N-methyl-2-methylquinolinium did not reduce ferricyanide in the presence of enzyme, but in this case a direct spectrophotometric assay could not be used, as the u.v. spectrum of the cation and its oxidation product are too similar. When tested using cytochrome c as electron acceptor, although rates were observed, they were too low to enable $K_m$ determination with either rabbit or guinea pig enzyme. [That oxidation of N-methyl-2-methylquinolinium does actually occur, was confirmed as aerobic incubation with aldehyde oxidase for one hour resulted in production of sufficient N-methyl-2-methyl-4-quinolone to allow identification. As expected when ferricyanide was added to the incubation mixture no oxidation product was obtained.] N-Methyl-2-methylquinolinium was not found to inhibit the oxidation of 6-methylpurine (monitored at 288 nm) to any appreciable extent; thus this cation does not interact particularly efficiently with aldehyde oxidase.

The $K_m$ values obtained for N-methyl-2-phenylquinolinium, N-methyl-4-phenylquinolinium and N-phenyl-2-phenylquinolinium indicate these compound to be good substrates for rabbit enzyme. However the rates of oxidation
of these cations with guinea pig enzyme were very slow (regardless of
electron acceptor employed) and insufficient to enable $K_m$ determinations
to be carried out, but even at low concentrations these cations were
found to inhibit the oxidation of other substrates. Hence $K_I$ values were
determined for N-methyl-2-phenylquinolinium, N-methyl-4-phenylquinolinium
and N-phenyl-2-phenylquinolinium against 6-methylpurine, as described
by the method outlined at the beginning of this section. The $1/v$ against
$1/s$ graphs (Figures 4.7b, 4.8b and 4.9) reveal these cations to be potent
competitive inhibitors of guinea pig enzyme.

The rates obtained for N-methylisoquinolinium with aldehyde oxidase
from either rabbit or guinea pig were insufficient to determine a $K_m$
value directly, regardless of electron acceptor employed. When this
compound was incubated with other substrates their rates of oxidation were
decreased but only at relatively high concentrations of N-methylisoquinolinium
This cation was found to competitively inhibit the oxidation of 6-methyl-
purine with either rabbit or guinea pig enzyme (see Figure 4.10a,b) and its
$K_I$ values are given in Table 4.1. From the results it would appear that
N-methylisoquinolinium is not a good substrate and only acts as an
inhibitor at relatively high concentrations.

\[ \text{Ethidium Bromide} \]
Ethidium bromide is an inhibitor of RNA and DNA synthesis\textsuperscript{225} and is used to treat sleeping sickness in cattle.\textsuperscript{226} It was not found to be a substrate for aldehyde oxidase from rabbit or guinea pig. This finding was not unexpected since a phenyl group occupies the position where hydroxylation of phenanthridinium compounds usually occurs. However the rate of oxidation of other substrates with aldehyde oxidase were greatly reduced in the presence of ethidium bromide, therefore indicating this compound to be an inhibitor. This inhibitory action was attributed to the ethidium cation rather than the bromide anion, since in control experiments rates of oxidation of substrates were unaffected by the presence of high concentrations (\textit{i.e.} $10^{-3}$ M) of sodium bromide. Ethidium bromide was found to be a potent competitive inhibitor of both guinea pig and rabbit enzyme and the $K_i$ values obtained, \textit{i.e.} $4.6 \times 10^{-6}$ M indicate the cation to be one of the best inhibitors so far known for aldehyde oxidase.

Many other chemotherapeutic agents used for treating sleeping sickness are N-heterocyclic cations substituted at positions where aldehyde oxidase may be expected to oxidise, \textit{e.g.}

\begin{center}
\begin{tikzpicture}
\node[anchor=north west, inner sep=0] (a) at (0,0) {Antrycide Chloride};
\begin{scope}[scale=0.8]
\draw[thick] (0,0) rectangle (2,2);
\node[anchor=north west, inner sep=0] (b) at (0.5,0.5) {$\text{NH}_2$};
\node[anchor=north west, inner sep=0] (c) at (1.5,0.5) {$\text{CH}_3$};
\node[anchor=north west, inner sep=0] (d) at (0.5,1.5) {$\text{NH}_2$};
\node[anchor=north west, inner sep=0] (e) at (1.5,1.5) {$\text{CH}_3$};
\draw[thick] (0,0.5) -- (1,0.5) -- (1,1.5) -- (0,1.5) -- cycle;
\draw[thick] (0,0) -- (0,1.5);
\draw[thick] (1,0) -- (1,1.5);
\draw[thick] (0.5,0.5) -- (0.5,1.5);
\draw[thick] (1.5,0.5) -- (1.5,1.5);
\fill[black] (0,0) circle (0.05);
\fill[black] (1,0) circle (0.05);
\fill[black] (0,1.5) circle (0.05);
\fill[black] (1,1.5) circle (0.05);
\end{scope}
\end{tikzpicture}
\end{center}

Tozocide

\begin{center}
\begin{tikzpicture}
\node[anchor=north west, inner sep=0] (a) at (0,0) {I$^-$ \textit{Hl}};
\begin{scope}[scale=0.8]
\draw[thick] (0,0) rectangle (2,2);
\node[anchor=north west, inner sep=0] (b) at (0.5,0.5) {$\text{NH}_2$};
\node[anchor=north west, inner sep=0] (c) at (1.5,0.5) {$\text{CH}_3$};
\node[anchor=north west, inner sep=0] (d) at (0.5,1.5) {$\text{NH}_2$};
\node[anchor=north west, inner sep=0] (e) at (1.5,1.5) {$\text{CH}_3$};
\draw[thick] (0,0.5) -- (1,0.5) -- (1,1.5) -- (0,1.5) -- cycle;
\draw[thick] (0,0) -- (0,1.5);
\draw[thick] (1,0) -- (1,1.5);
\draw[thick] (0.5,0.5) -- (0.5,1.5);
\draw[thick] (1.5,0.5) -- (1.5,1.5);
\fill[black] (0,0) circle (0.05);
\fill[black] (1,0) circle (0.05);
\fill[black] (0,1.5) circle (0.05);
\fill[black] (1,1.5) circle (0.05);
\end{scope}
\end{tikzpicture}
\end{center}
It would be interesting to investigate whether these compounds can act as inhibitors of aldehyde oxidase.

\( K_m \) determinations using cytochrome c

A \( K_m \) value of \( 1 \times 10^{-4} \text{ M}^3 \) was previously reported for the oxidation of \( N \)-methylquinolininium with rabbit aldehyde oxidase using cytochrome c, which is a much lower value than found in the present investigation using ferricyanide. It was suspected that this variation may be due to the nature of the electron acceptor employed and the \( K_m \) values for the compounds listed in Table 4.2 were therefore redetermined using cytochrome c.

<table>
<thead>
<tr>
<th>Source of aldehyde oxidase</th>
<th>(i) with cytochrome c</th>
<th>(ii) with ferricyanide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quinoline</td>
<td>R</td>
<td>1.3 \times 10^{-3} (0.995)</td>
</tr>
<tr>
<td>N-Methylquinolininium</td>
<td>R</td>
<td>2.3 \times 10^{-4} (0.996)</td>
</tr>
<tr>
<td></td>
<td>GP</td>
<td>5.0 \times 10^{-4} (0.998)</td>
</tr>
<tr>
<td>N-Phenylquinolininium</td>
<td>R</td>
<td>5.6 \times 10^{-6} (0.995)</td>
</tr>
<tr>
<td></td>
<td>GP</td>
<td>2.2 \times 10^{-5} (0.998)</td>
</tr>
</tbody>
</table>

\( a \) Values in parentheses are correlation figures

R = rabbit  GP  = guinea pig

Table 4.2 shows that the difference in \( K_m \) values obtained with the two electron acceptors is largest for the quaternised compounds with rabbit enzyme, but not as marked for the uncharged substrate quinoline. \( K_m \) values obtained with ferricyanide were consistently higher than those obtained with cytochrome c. A similar discrepancy between these two electron acceptors was noted by Palmer using crotonaldehyde as a
substrate \( K_m \) with ferricyanide was reported to be \( 6.7 \times 10^{-3} \) M but only \( 1.2 \times 10^{-3} \) M with cytochrome \( c \). However, regardless of electron acceptor employed it can be seen that quaternisation of quinoline by either a methyl or phenyl group enhances its susceptibility to enzymatic oxidation.

It was found that the choice of electron acceptor had an even greater effect on \( V_{\text{max}} \) than \( K_m \) values. For N-methylquinolinium perchlorate, the value obtained with cytochrome \( c \) was only 7% of that found using ferricyanide for both sources of enzyme. Likewise, for N-phenylquinolinium, the \( V_m \) value obtained using cytochrome \( c \) was only 3.5% of that found using ferricyanide. In this case a similar effect was also noted with the uncharged substrate quinoline, which is oxidised at a much slower rate than either of the above quaternised derivatives and gave a \( V_{\text{max}} \) value with cytochrome \( c \) which was only 17% of that obtained with ferricyanide.

The reason for such differences probably lies in the way cytochrome \( c \) functions as an electron acceptor in this system. In the presence of oxygen two possible pathways of reoxidation of the enzyme have been postulated, \(^{126,128}\) viz.

\[
\text{Enzyme-}H_2 + 2O_2 \rightarrow \text{Enzyme} + 2H^+ + 2O_2^- \quad (1a)
\]

\[
2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2 \quad (1b)
\]

\[
\text{Enzyme-}H_2 + O_2 \rightarrow \text{Enzyme} + H_2O_2 \quad (2)
\]

Cytochrome \( c \) is reduced only by the superoxide anions resulting from the univalent reduction of oxygen \( (1a) \).

\[
\text{Cyt } c^{+++} + O_2^- \rightarrow \text{Cyt } c^{++} + O_2
\]

Therefore, cytochrome \( c \) is only monitoring a percentage of the total
electron flux and this percentage has been Previously shown to vary according to pH, oxygen tension and the rate of turnover of the enzyme.\textsuperscript{126,130} Fridovich\textsuperscript{126} found that at pH 7, in air, only 20% of the total electron flux through xanthine oxidase can be accounted for in terms of univalent reduction of oxygen. In agreement, the rate of reduction of cytochrome c was found to be 22% of the rate of urate production from xanthine. Fridovich also postulated that at high turnover rates of the enzyme, dismutation of O\textsubscript{2} produced competed with its ability to reduce cytochrome c, which was suggested to account for the lower $K_m$ obtained for xanthine using this electron acceptor (0.61 x 10\textsuperscript{-6} M) compared with the value obtained by directly monitoring product (1.5 x 10\textsuperscript{-6} M).

Therefore the cytochrome c assay is not very satisfactory because of the number of variables that may influence it.

4.3 Comparison of Michaelis Constants ($K_m$ and $K_o$ Values)

One of the most interesting findings in this present study is the ability of aldehyde oxidase to oxidise a number of quinolinium compounds at carbon 4. Previously oxidation at a carbon removed from nitrogen was considered unusual and hence one may have predicted the $K_m$ values for 4-quinolone formation to be high. However, using rabbit enzyme, a reasonable $K_m$ value was obtained for N-methyl-2-phenylquinolinium (oxidised exclusively at carbon 4) which is comparable with that found for N-methyl-4-phenylquinolinium (oxidised at carbon 2). Furthermore, N-phenyl-2-phenylquinolinium has a $K_m < 10^{-5}$ M making it one of the best substrates known for aldehyde oxidase.

Guinea pig liver aldehyde oxidase was generally found to have a greater affinity for N-heterocyclic cations than rabbit enzyme as shown in Table 4.1. Guinea pig enzyme catalysed the oxidation of N-methyl-
quinolinium and N-phenylquinolinium but substitution of a phenyl group at positions 2 or 4 resulted in compounds which were only extremely slowly oxidised; they were however found to be potent competitive inhibitors. For example, the $K_i$ for N-phenyl-2-phenylquinolinium is $8 \times 10^{-7}$ M. In comparison these compounds were readily oxidised by rabbit enzyme.

From the results in Table 4.1 it appears that the ability of either enzyme to bind N-heterocyclic cations generally increases with increase in hydrophobic nature of the compound. For example, addition of a further benzene ring or phenyl group to N-methylquinolinium increased the affinity, i.e., decreased the $K_i$ or $K_m$ value obtained; the most effective binders were found to be the tetracyclic cations N-phenyl-2-phenyl-quinolinium and ethidium bromide. Furthermore, the monocyclic cation N-methylpyridinium has been reported to be neither a good substrate nor good inhibitor ($K_i = 1.9 \times 10^{-2}$ M) of aldehyde oxidase. These findings imply that aldehyde oxidase has a large binding site and also suggests that there are regions of considerable hydrophobicity in which annelated aromatic rings bind.

Comparison of Michaelis constants for quaternary and non-quaternary compounds with rabbit enzyme

Because of their greater susceptibility to nucleophilic attack, quaternised compounds are generally considered to be better substrates for aldehyde oxidase than their unquaternised counterparts. Thus as shown in Table 4.2 the $K_m$ values for N-methyl and N-phenylquinolinium salts are lower than the $K_m$ value for quinoline. In addition, whereas 5,6- and 7,8-benzoquinolines were reported to be neither substrates nor inhibitors of aldehyde oxidase, their N-methylated derivatives were found to be good substrates. However, two important exceptions to this generalisation were noted. Phenanthridine is an excellent substrate for
aldehyde oxidase ($K_m < 10^{-6}$ M) but its N-methylated analogue is somewhat less efficient in this respect. Furthermore, whereas isoquinoline is a much better substrate than the isomeric quinoline (being oxidised at ca. twice the rate; having a $K_m$ value of $1.96 \times 10^{-4}$ M compared to $3 \times 10^{-3}$ M for quinoline), N-methylisoquinolinium was found to be neither a good substrate nor a good inhibitor. Thus the relationship between N-heterocyclic cations and their parent unquaternised compounds with aldehyde oxidase is not as simple as originally envisaged. Perhaps this is not too surprising since factors other than nucleophilicity would be expected to play a role in the enzymatic oxidation of substrates. It is interesting to note that a previous investigation of the oxidation of 7-aminothiadiazolo[3,4-d]pyrimidine (1a) and of 7-aminofurazano[3,4-d]-pyrimidine (1b) with aldehyde oxidase found the $K_m$ values and maximal velocities to be very similar for both compounds despite the substantially greater susceptibility of compound 1b to undergo nucleophilic attack at position 5 (the position of enzymatic oxidation).

At present it is difficult to explain why N-methylisoquinolinium is refractory to enzymatic oxidation, but it is noted that this cation bears the same benzologous relationship to the N-methylphenantridinium cation (substrate) that the 1-methylpyridinium cation (non-substrate) bears to
the 1-methylquinolinium cation (substrate) as shown below:

This seems to suggest that the interaction between the homocyclic-ring (shaded in the figure above) and the enzyme is important in determining the binding of these substrates at the active site. However this speculative "binding picture" cannot be applied to the non-quaternary compounds since it does not explain why isoquinoline is a better substrate than quinoline. Furthermore it has been reported\(^6\) that whilst phenanthridine is an excellent substrate, the isomers 5,6- and 7,8-benzoquinolines do not bind to the enzyme, it was suggested that the latter compounds did not "fit" into the active site. However it is difficult to reconcile this with the fact that the N-methylated derivatives are all good substrates (similar $K_m$ values were obtained).
This raises two questions:

(i) do quaternary and non-quaternary compounds bind similarly, or
(ii) do these two classes of compound bind at different sites on the enzyme?

These questions will be discussed in sections 4.6 and 4.7.

4.4 Comparison of Rates of Oxidation

Table 4.3 gives the rates of oxidation of a range of quaternary and non-quaternary compounds with guinea pig and rabbit aldehyde oxidases and bovine milk xanthine oxidase measured under the following conditions:

(i) Rates were monitored by following the reduction of the electron acceptor potassium ferricyanide at 420 nm, using the method outlined in section 2.6.2.1. Thus the absorbance change per mole of substrate oxidised is the same for every substrate tested.

(ii) The substrate concentration used was ca. 10-20 x $K_m$, where known, and thus it is assumed that the rates obtained are the maximal velocities ($V_{max}$ values). In each case, rates were also measured at lower substrate concentrations to see if any substrate inhibition was occurring at the higher values. None of the compounds tested showed this effect.

(iii) The rates were determined using the same batch of each particular enzyme and were adjusted to correspond to 1 mg of protein per assay. Partially purified aldehyde oxidase was used. Since guinea pig and rabbit enzyme was similarly prepared, it is reasonable to directly compare rates between these two enzymes. Commercially available milk xanthine oxidase was used.

It is often assumed that a faster relative rate corresponds to a lower dissociation constant, i.e. the faster the rate of reaction, the better the substrate binds. However throughout this study many exceptions
Table 4.3. Relative rates of oxidation of a number of compounds with rabbit liver aldehyde oxidase, guinea pig liver aldehyde oxidase and milk xanthine oxidase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Substrate conc/M</th>
<th>Rabbit Aldehyde Oxidase</th>
<th>Guinea Pig Aldehyde Oxidase</th>
<th>Milk Xanthine Oxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quinoline</td>
<td>$1 \times 10^{-2}$</td>
<td>19</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>3-Methylquinoline</td>
<td>$5 \times 10^{-3}$</td>
<td>46</td>
<td>14</td>
<td>b</td>
</tr>
<tr>
<td>4-Methylquinoline</td>
<td>$5 \times 10^{-3}$</td>
<td>12</td>
<td>44</td>
<td>b</td>
</tr>
<tr>
<td>Isoquinoline</td>
<td>$1 \times 10^{-3}$</td>
<td>42</td>
<td>40</td>
<td>b</td>
</tr>
<tr>
<td>3-Methylisoquinoline</td>
<td>$5 \times 10^{-4}$</td>
<td>43</td>
<td>23</td>
<td>b</td>
</tr>
<tr>
<td>Phenanthridine</td>
<td>$5 \times 10^{-5}$</td>
<td>164</td>
<td>106</td>
<td>b</td>
</tr>
<tr>
<td>5,6-Benzoquinoline</td>
<td>$1 \times 10^{-3}$</td>
<td>3</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>7,8-Benzoquinoline</td>
<td>$5 \times 10^{-4}$</td>
<td>b</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>Phthalazine</td>
<td>$1 \times 10^{-4}$</td>
<td>296</td>
<td>424</td>
<td>5</td>
</tr>
<tr>
<td>Quinazoline</td>
<td>$1 \times 10^{-4}$</td>
<td>240</td>
<td>467</td>
<td>25</td>
</tr>
<tr>
<td>6-Methylpurine</td>
<td>$1 \times 10^{-2}$</td>
<td>100</td>
<td>212</td>
<td>3</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>$1 \times 10^{-2}$</td>
<td>108</td>
<td>261</td>
<td>14</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>$1 \times 10^{-3}$</td>
<td>224</td>
<td>467</td>
<td>40</td>
</tr>
<tr>
<td>N-Methylquinolinium</td>
<td>$1 \times 10^{-2}$</td>
<td>120</td>
<td>212</td>
<td>b</td>
</tr>
<tr>
<td>N-Methyl-2-methylquinolinium</td>
<td>$2 \times 10^{-3}$</td>
<td>42</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>N-Methyl-4-methylquinolinium</td>
<td>$1 \times 10^{-3}$</td>
<td>65</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>N-Phenylquinolinium</td>
<td>$1 \times 10^{-3}$</td>
<td>240</td>
<td>354</td>
<td>b</td>
</tr>
<tr>
<td>N-Phenyl-2-phenylquinolinium</td>
<td>$1 \times 10^{-4}$</td>
<td>64</td>
<td>7</td>
<td>b</td>
</tr>
<tr>
<td>N-Methylisoquinolinium</td>
<td>$2 \times 10^{-2}$</td>
<td>b</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>N-Methylphenanthridinium</td>
<td>$2 \times 10^{-3}$</td>
<td>120</td>
<td>81</td>
<td>b</td>
</tr>
<tr>
<td>N-Methyl-5,6-benzoquinolinium</td>
<td>$2 \times 10^{-3}$</td>
<td>38</td>
<td>81</td>
<td>b</td>
</tr>
<tr>
<td>N-Methyl-7,8-benzoquinolinium</td>
<td>$2 \times 10^{-3}$</td>
<td>70</td>
<td>22</td>
<td>b</td>
</tr>
</tbody>
</table>

*a* Rates obtained using 1 mg protein. Rate of 10 corresponds to 6.27 nmoles of substrate oxidised min$^{-1}$ at 30°C and pH 7

*b* Negligible or no rate observed

*c* Higher concentrations could not be used because of the insolubility of the compounds

*d* $2 \times 10^{-4}$ M used for guinea pig enzyme
to this assumption were noted. For example:

(i) With guinea pig enzyme the $K_m$ value for N-methyl-7,8-benzoquinolinium is seven times lower than that for N-methylquinolinium but the maximal velocity for the latter compound is ten times faster than that of the former compound.

(ii) With rabbit enzyme the maximal velocity for N-phenyl-2-phenylquinolinium is half that obtained with N-methylquinolinium despite the former compound binding by a factor of at least 100 times more efficiently.

These exceptions were not restricted to quaternary compounds, for example:

(iii) Isoquinoline has a ten times greater affinity for rabbit enzyme than 6-methylpurine although 6-methylpurine is oxidised twice as fast as isoquinoline.

Davis\(^2^{28}\) stated that there is no justification for the assumption above, since with many substrates it is the release of product which is the rate limiting step, implying that the relative rate is independent of enzyme-substrate interaction. Therefore many of the conclusions predicted upon this correlation between activity and affinity must be re-evaluated.

Menadione is a potent inhibitor of aldehyde oxidase.\(^5\) The site of interaction of menadione on the enzyme is not clear, but it is not considered to be the active site, rather it inhibits the internal electron transport chain.\(^2^{29}\) Johns\(^9\) reported that with human liver enzyme whilst menadione inhibited the oxidation of uncharged substrates, it did not inhibit the oxidation of N-heterocyclic cations. The rates of all the compounds in Table 4.3 with aldehyde oxidase were repeated under the same conditions, but in the presence of $5 \times 10^{-5}$ M menadione. The rates were all reduced to zero, with one exception: the rate obtained for N-phenyl-2-phenylquinolinium with guinea pig enzyme was only reduced by 50% in the presence of menadione. This may be related to the fact that this compound
has an extremely high affinity for the enzyme. Thus it can be concluded that menadione inhibited the rates of oxidation of both quaternary and non-quaternary compounds with aldehyde oxidase from both rabbit and guinea pig.

Generally the oxidation of \( N \)-heterocyclic cations with xanthine oxidase is not considered to take place below pH 9.\textsuperscript{33,34} However one exception has been noted by Bergmann \textit{et al.}\textsuperscript{36}

\[
\begin{array}{c}
\text{H}_3\text{C}-\text{N} \\
\text{N} \\
\text{N} \\
\text{N} \\
\text{CH}_3 \\
\end{array}
\xrightarrow{\text{X.O.}}
\begin{array}{c}
\text{H}_3\text{C}-\text{N} \\
\text{N} \\
\text{N} \\
\text{N} \\
\text{CH}_3 \\
\end{array}
\]

Conversion of the hypoxanthine (A) to the 2-pyridone (B) was found to be faster at pH 8 than at pH 10 or 11. In keeping with the general rule however, none of the \( N \)-heterocyclic cations studied in this present investigation showed any activity with xanthine oxidase at pH 7 (Table 4.3). Bunting \textit{et al.}\textsuperscript{34} reported that at alkaline pH, \( N \)-methyl quinolinium, phenanthridium and benzoquinolinium cations were substrates for xanthine oxidase although \( N \)-methylisoquinolinium was not. These observations are similar to those for aldehyde oxidase at pH 7. In the present study, an important specificity difference between these two enzymes, with respect to \( N \)-heterocyclic cations, was found, \textit{i.e.} whereas ethidium bromide is an excellent inhibitor of aldehyde oxidase it did not inhibit xanthine oxidase even at pH 10. Of all the compounds tested, only the uncharged
substrates quinazoline and acetaldehyde were rapidly oxidised by all the three enzymes studied.

This rate study again highlights the differences that exist, not only between aldehyde oxidase and xanthine oxidase, but also between different sources of aldehyde oxidase. It has been postulated that xanthine oxidase and aldehyde oxidase have a common evolutionary origin;\(^\text{19}\) furthermore it has been argued\(^\text{46}\) that because of the greater variation of the latter enzyme from different species, this is the one in transition.

4.5 **Simultaneous Formation of 2- and 4-Quinolones: A Study of Product Ratios Under Various Conditions**

In Chapter 3 (section 3.4), aldehyde oxidase was identified as the enzyme responsible for the production of both 2- and 4-quinolones from N-methyl and N-phenylquinolinium salts. Simultaneous formation of two products from one substrate is unusual and raises the following questions:

(i) Does different orientation of the substrate molecule at a *single* active site on the enzyme account for formation of the two isomeric products?

(ii) Is there more than one type of active site on the same enzyme molecule?

(iii) Are isoenzymes present?

(iv) Is some other factor, perhaps related to electronic distribution in the substrate molecule, important?

Studying product ratios under a variety of conditions including the use of selective inhibitors or inducers might give some indication as to which of these possibilities is most likely.\(^\text{230,231}\)

Several conditions have already been discussed which were shown to have no effect on product ratios. These included: variation of substrate concentration, purification of the enzyme, the use of ferri-
cyanide as the electron acceptor, anaerobic incubations and addition of catalase to the system (see section 3.4). In addition, storage of the enzyme in liquid nitrogen does not alter product ratio.

Aldehyde oxidase is susceptible to poisoning by heavy metals but the metal chelating agent EDTA protects against such inactivation. Thus buffer systems used in this study usually contained $10^{-4}$ M EDTA. Variation of the EDTA concentration from $5 \times 10^{-5}$ M to $10^{-2}$ M had no effect on either the amount or ratio of 2- and 4-quinolone formation.

It has recently been found that the anti-hypertensive drug hydralazine is a potent inhibitor of aldehyde oxidase in vitro and that enzyme preparations from rabbit treated with this drug also showed a marked decrease in aldehyde oxidase activity. Furthermore, the same authors found that phthalazine (5 mg/kg for 21 days) appeared to cause induction of the enzyme in vivo. Partially purified aldehyde oxidase from the livers of rabbits treated with either hydralazine or phthalazine still gave the same product ratio with N-methylquinolinium perchlorate as that from control animals.

The effect of pH on the 2- to 4-quinolone ratio was investigated for N-methylquinolinium perchlorate with rabbit enzyme (for experimental see section 2.4.2).

<table>
<thead>
<tr>
<th>pH</th>
<th>Ratio 2:4 quinolone (±1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.3</td>
<td>19</td>
</tr>
<tr>
<td>6.1</td>
<td>18</td>
</tr>
<tr>
<td>7.0</td>
<td>17</td>
</tr>
<tr>
<td>7.7</td>
<td>18</td>
</tr>
<tr>
<td>8.7</td>
<td>14</td>
</tr>
<tr>
<td>9.3</td>
<td>13</td>
</tr>
<tr>
<td>9.7</td>
<td>14</td>
</tr>
</tbody>
</table>

*Buffer system used as in section 2.6.2.4.*
The results in Table 4.4 were obtained using G75 fraction enzyme and show a tendency to produce a greater proportion of the 4-quinolone at higher pH values. G75 enzyme contains some xanthine oxidase activity and since Greenlee and Handler found milk xanthine oxidase to oxidise N-methylquinolinium to N-methyl-2-quinolone at high pH values; it was considered that the presence of this enzyme in aldehyde oxidase preparations may affect product ratios. However even after further purification of the enzyme by passing down a DEAE column (which removes any xanthine oxidase) repetition of the pH study gave similar results to those obtained with the G75 fraction.

Previous investigations of the production of 2- and 4-pyridones from N'-methylnicotinamide also showed change in product ratio with pH. For example, the ratio of 2- to 4-pyridone formation with partially purified mouse liver aldehyde oxidase was found to increase from 0.8 at pH 6.4 to 2.88 at pH 9.8, which is a much greater change than was observed for N-methylquinolinium and indicates that a greater proportion of 2-pyridone is formed at higher pH values. However as was discussed by Porter et al., one cannot conclude from variation of ratios with pH that the substrate is binding at more than one enzyme site because it is possible that this variation may be due to ionisation changes at a single active site, affecting orientation of the substrate at that site.

The oxidation of N-methylquinolinium perchlorate (2.5 x 10^{-3} M) with rabbit liver aldehyde oxidase was monitored in the presence of the competing substrate, N-methylphenanthridinium perchlorate (10^{-3} M). The latter compound was chosen because oxidation only occurs at one position, that is, at the carbon adjacent to the nitrogen atom. Therefore it might be expected that this compound would preferentially inhibit 2-quinolone formation, if two different enzymatic sites are present. Surprisingly, the converse proved to be the case, the ratio of 2- to 4-quinolone increased
from 17(±1) (absence of competing substrate) to 29(±2) in the presence of N-methylphenanthridinium, indicating that 4-quinolone production is inhibited more effectively than that of the 2-quinolone. This observation may suggest the presence of two enzymatic sites with differing affinities for the substrate N-methylphenanthridinium.

Thus in summary, the product ratio obtained from N-methylquinolinium salts was found to be constant under various conditions, including purification of the enzyme and the use of either induced or inhibited aldehyde oxidase but a change in the ratio was found at high pH and in the presence of the competing substrate, N-methylphenanthridinium. These results are similar to those previously obtained by Felsted and Chaykin for the oxidation of N1-methylnicotinanide with aldehyde oxidase. They found that, of the conditions studied, only variation of pH and presence of the inhibitor Amytal resulted in a change in the ratio of 2- to 4-pyridone formation. It was suggested that these findings may be indicative of either different binding conformations of the substrate being required for the two oxidations or different oxidation sites on the enzyme or both; then, titration or Amytal blockage of a critical group could be visualised as differentially affecting the binding or catalysis which leads to the formation of the oxidation products.

Using N-methyl-4-phenylquinolinium perchlorate, which is oxidised exclusively at carbon 2, and N-methyl-2-phenylquinolinium perchlorate which is oxidised exclusively at carbon 4, a kinetic study was devised to determine whether 2-quinolone and 4-quinolone formation occurs at the same site on the enzyme.

The rate of oxidation of N-methyl-4-phenylquinolinium perchlorate was monitored by the corresponding decrease in absorbance at 367 nm; at this wavelength no significant change in absorbance was observed during
the oxidation of N-methyl-2-phenylquinolinium. Thus it was possible to monitor the effect of the latter substrate on the rate of oxidation of N-methyl-4-phenylquinolinium perchlorate. The rates for a range of concentrations of N-methyl-4-phenylquinolinium perchlorate were determined using rabbit liver aldehyde oxidase, both with and without the addition of N-methyl-2-phenylquinolinium perchlorate.

A $1/v$ versus $1/s$ plot obtained from these results is shown in Figure 4.13 and demonstrates quite clearly that N-methyl-2-phenylquinolinium competitively inhibits the oxidation of N-methyl-4-phenylquinolinium. The $K_i$ value for the competing substrate N-methyl-2-phenylquinolinium was calculated to be $5 \times 10^{-4}$ M which is in very good agreement with its $K_m$ value of $4.6 \times 10^{-4}$ M. These results suggest that binding of N-methyl-4-phenylquinolinium and N-methyl-2-phenylquinolinium, and consequently that formation of both 2-quinolones and 4-quinolones, occur at the same site on the enzyme.

4.6 Quaternary and Non-quaternary Compounds: A Binding Site Investigation

4.6.1 Inhibition studies

Table 4.1 shows the $K_i$ values determined for some N-heterocyclic cations. These values were determined from inhibition studies using 6-methylpurine. In each case the inhibition was shown to be competitive. From these results it would appear that both quaternary cations and the non-quaternary compound 6-methylpurine act at a common site on the enzyme. Moreover the $K_i$ for N-methylisoquinolinium was found to be the same, whether measured against 6-methylpurine or the quaternary compound N-methyl-2-phenylquinolinium with rabbit enzyme.

Further evidence for a common site came from studying the inhibition of the oxidation of 6-methylpurine by N-methyl-2-phenylquinolinium with
Figure 4.13. Inhibition by the competing substrate N-methyl-2-phenylquinolinium of the rabbit aldehyde oxidase catalysed oxidation of N-methyl-4-phenylquinolinium

Oxidation of N-methyl-4-phenylquinolinium (○) and in the presence of 8.6 x 10^{-4} M (●), and 1.2 x 10^{-4} M (▲) N-methyl-2-phenylquinolinium perchlorate.
rabbit enzyme. Both of these compounds are good substrates, which is a different situation from the above inhibition studies where oxidation of the N-heterocyclic cation was negligible. The rate of oxidation of 6-methylpurine was monitored by the corresponding increase in absorbance at 288 nm (Δε = 3,090); at this wavelength no significant change in absorbance was observed for the oxidation of N-methyl-2-phenylquinolinium to N-methyl-2-phenyl-4-quinolone. Thus it was possible to monitor the effect of this substrate on the rate of oxidation of 6-methylpurine. The rates for a range of concentrations of 6-methylpurine were determined both with and without the addition of 4.5 × 10^{-4} M N-methyl-2-phenylquinolinium perchlorate. A 1/v versus 1/s plot was obtained from these results as shown in Figure 4.14 which demonstrates the competitive nature of the inhibition of the oxidation of 6-methylpurine by N-methyl-2-phenylquinolinium. The $K_z$ value calculated for this cation was 2.2 × 10^{-4} M which is in reasonable agreement with its $K_m$ value of 4.6 × 10^{-4} M.

From these results it would appear that quaternary cations, regardless of whether they are oxidised in either the C-2 or C-4 position, act at the same active site on the enzyme as non-quaternary compounds.

4.6.2 Effect of pH on substrate specificity

The effect of pH on enzymes, like all pH effects, is due to changes in the state of ionisation of the components of the system as the pH changes. Either the free enzyme, the enzyme-substrate complex (E-S) or the substrate may undergo such changes. The effect of pH on $K_m$ depends on the ionisation constants of all three components, while $V_{\text{max}}$ is a function of the constants of the E-S complex only; in the ratio of the two quantities ($V_{\text{max}} / K_m$) the E-S constants are cancelled out, leaving only the constants of the free enzyme and substrate.

Since enzymes are proteins containing many ionisable groups, they exist in a whole series of different states of ionisation and the
Figure 4.14. Inhibition by the competing substrate, N-methyl-2-phenylquinolinium, of the rabbit aldehyde oxidase catalysed oxidation of 6-methylpurine.

Oxidation of 6-methylpurine (○) and in the presence of $4.5 \times 10^{-4}$ M N-methyl-2-phenylquinolinium (●).
distribution of the enzyme among the various ionic forms depends on the pH and the ionisation constants of the various groups. However it is assumed that catalytic activity is greatly affected by the ionisation state of group(s) at the active centre of the enzyme but that ionisation of groups in the protein which are remote from the active centre have little or no effect.  

A detailed study was made of the variation of $V_{\text{max}}$ and $K_m$ values over the pH range 5.5 to 10, with a non-quaternary compound and with N-heterocyclic cations. The experimental details are given in section 2.6.2.4. Previously it was reported that with increasing pH, $K_m$ values for compounds bearing a positive nitrogen decrease while the $K_m$ values for non-quaternary substrates increase. It was hoped that a more thorough investigation may yield the ionisation constants for the group or groups on the free enzyme involved in substrate binding.

4.6.2.1 pH profile for the oxidation of phthalazine

Phthalazine is a suitable substrate for monitoring pH effects for the following reasons:

(i) It has a $pK_a$ of 3.46, therefore no change in ionisation of this compound occurs in the pH range under investigation. This also simplifies analysis of $\log(V_{\text{max}}/K_m)$ values with pH, as any changes must reflect ionisation changes of the free enzyme alone.

(ii) This compound is stable over the pH range studied.

(iii) Phthalazine has previously been shown to be a good substrate for aldehyde oxidase with a $K_m$ of $9.9 \times 10^{-5}$ M at pH 7, and
produces only one oxidation product, i.e. 1-hydroxyphthalazine.

(iv) The poor solubility of many non-quaternary substrates restricts the range of concentrations that could be studied; however phthalazine is reasonably soluble.

The results of the oxidation of phthalazine with rabbit liver aldehyde oxidase at ten pH values is given in Table 4.5. Between six and ten data points were used for each $K_m$ determination ($v$ and $s$ values were fed into a computer programme which calculated $K_m$ and $V_{max}$ for the substrate, using the Lineweaver-Burk transformation of the Michaelis-Menten equation).

Table 4.5. Variation of $K_m$ and $V_{max}$ values for phthalazine with pH

<table>
<thead>
<tr>
<th>pH</th>
<th>$K_m$ a/M</th>
<th>$V_{max}$ b</th>
<th>log($V_{max}/K_m$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.8</td>
<td>$4.47 \times 10^{-5}$ (0.996)</td>
<td>0.150</td>
<td>3.53</td>
</tr>
<tr>
<td>6.1</td>
<td>$5.21 \times 10^{-5}$ (0.999)</td>
<td>0.230</td>
<td>3.64</td>
</tr>
<tr>
<td>6.6</td>
<td>$5.67 \times 10^{-5}$ (0.991)</td>
<td>0.290</td>
<td>3.70</td>
</tr>
<tr>
<td>7.1</td>
<td>$1.15 \times 10^{-4}$ (0.999)</td>
<td>0.416</td>
<td>3.56</td>
</tr>
<tr>
<td>7.5</td>
<td>$1.54 \times 10^{-4}$ (0.997)</td>
<td>0.460</td>
<td>3.48</td>
</tr>
<tr>
<td>8.1</td>
<td>$3.02 \times 10^{-4}$ (0.999)</td>
<td>0.380</td>
<td>3.10</td>
</tr>
<tr>
<td>8.5</td>
<td>$5.20 \times 10^{-4}$ (0.997)</td>
<td>0.317</td>
<td>2.79</td>
</tr>
<tr>
<td>9.0</td>
<td>$5.85 \times 10^{-4}$ (0.996)</td>
<td>0.298</td>
<td>2.70</td>
</tr>
<tr>
<td>9.3</td>
<td>$8.76 \times 10^{-4}$ (0.997)</td>
<td>0.246</td>
<td>2.45</td>
</tr>
<tr>
<td>9.6</td>
<td>$1.20 \times 10^{-3}$ (0.995)</td>
<td>0.176</td>
<td>2.17</td>
</tr>
</tbody>
</table>

a Figures in parentheses are correlation values
b Units are µmol substrate/min/mg protein (see section 2.6.3)

A twenty-five fold increase in $K_m$ was observed with increase in pH from 5.8 to 9.6. However even at pH 9.6 phthalazine can still be
regarded as a reasonable substrate, with its $K_m$ value ($1.2 \times 10^{-3}$ M) comparable to that for 6-methylpurine at pH 7 ($1.5 \times 10^{-3}$ M). In comparison the $V_{max}$ values were found to vary little with pH (viz. from 0.15 to 0.46), with maximum activity occurring at ca. pH 7.5.

A log($V_{max}/K_m$) versus pH graph was plotted from the results in Table 4.5 (see Figure 4.15). From pH 5.8 to 7.5 the values of log($V_{max}/K_m$) did not alter very much but decreased sharply at higher pH values. It has been shown that variation of log($V_{max}/K_m$) values with pH are due to changes in ionisation of groups at the active site of the free enzyme. Furthermore from these plots $pK$ value(s) of the free enzyme can be determined since a bend in the curve indicates the $pK$ of an ionising group. The straight portions of the curve are extrapolated and intersect at a pH corresponding to the $pK$. From Figure 4.15 it would appear that rabbit liver aldehyde oxidase has a group at its active site with a $pK$ of ~7.7 which is important for binding phthalazine. However, the straight portion of the curve, which should in theory have integral slope (i.e. zero or one-unit or two-unit), only had a slope of 0.7. The significance of this, and the validity and interpretation of these results, will be discussed in section 4.6.2.5.

**4.6.2.2 pH profile for the oxidation of N-methyl-2-phenylquinolinium perchlorate**

The results of oxidation of N-methyl-2-phenylquinolinium perchlorate with rabbit liver aldehyde oxidase at eleven pH values is given in Table 4.6. This compound does not undergo any change in ionisation in the pH range studied and is oxidised exclusively at the 4 position.

* Also due to changes in ionisation of the substrate, but this is not considered since the substrates studied do not undergo any changes in ionisation over the pH range investigated.
Figure 4.15. Plot of $\log(V_{\max}/K_m)$ versus pH for phthalazine with rabbit aldehyde oxidase.
Table 4.6. Variation of $K_m$ and $V_{max}$ values for N-methyl-2-phenyl quinolinium with pH

<table>
<thead>
<tr>
<th>pH</th>
<th>$K_m^a$/M</th>
<th>$V_{max}^b$</th>
<th>$\log(V_{max}/K_m)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.82</td>
<td>$1.05 \times 10^{-3}$ (0.995)</td>
<td>0.0136</td>
<td>1.11</td>
</tr>
<tr>
<td>6.47</td>
<td>$5.69 \times 10^{-4}$ (0.996)</td>
<td>0.0326</td>
<td>1.76</td>
</tr>
<tr>
<td>6.85</td>
<td>$3.07 \times 10^{-4}$ (0.998)</td>
<td>0.0435</td>
<td>2.15</td>
</tr>
<tr>
<td>7.20</td>
<td>$3.48 \times 10^{-4}$ (0.998)</td>
<td>0.0650</td>
<td>2.27</td>
</tr>
<tr>
<td>7.44</td>
<td>$2.89 \times 10^{-4}$ (0.998)</td>
<td>0.080</td>
<td>2.44</td>
</tr>
<tr>
<td>7.83</td>
<td>$1.92 \times 10^{-4}$ (0.995)</td>
<td>0.135</td>
<td>2.85</td>
</tr>
<tr>
<td>8.00</td>
<td>$1.12 \times 10^{-4}$ (0.997)</td>
<td>0.081</td>
<td>2.86</td>
</tr>
<tr>
<td>8.74</td>
<td>$1.26 \times 10^{-4}$ (0.999)</td>
<td>0.181</td>
<td>3.16</td>
</tr>
<tr>
<td>8.95</td>
<td>$1.11 \times 10^{-4}$ (0.999)</td>
<td>0.169</td>
<td>3.18</td>
</tr>
<tr>
<td>9.22</td>
<td>$1.19 \times 10^{-4}$ (0.999)</td>
<td>0.192</td>
<td>3.21</td>
</tr>
<tr>
<td>9.70</td>
<td>$1.06 \times 10^{-4}$ (0.999)</td>
<td>0.200</td>
<td>3.28</td>
</tr>
</tbody>
</table>

$^a$ Figures in parentheses are correlation values

$^b$ Units are µmol substrate/min/mg

A ten-fold decrease in $K_m$ was observed with increase in pH from 5.82 to 9.70. The $V_{max}$ values were generally found to increase with increase in pH. A $\log(V_{max}/K_m)$ versus pH graph was plotted from these results (see Figure 4.16). From pH 5.82 to 8 the values of $\log(V_{max}/K_m)$ increased but did not alter very much at high pH values. From this particular plot, it would appear that rabbit liver aldehyde oxidase has a group at its active site with a pK of ≈7.9 which is important for binding N-methyl-2-phenylquinolinium perchlorate. Variation of pH similarly affected the oxidation of the N-heterocyclic cation, N-methylquinolinium, as discussed in section 4.6.2.4.
Figure 4.16. Plot of log($V_{max}/K_m$) versus pH for n-methyl-α-phenylglutathionyltransferase with rabbit aldehyde oxidase.
As expected, with increase in pH the \( K_m \) values for the quaternary compound N-methyl-2-phenylquinolinium were found to decrease while those values obtained for the non-quaternary compound, phthalazine, increased. Maximal activity of the enzyme with phthalazine occurred at \( \approx \text{pH 7.5} \); in contrast the \( V_{\text{max}} \) values obtained with the quaternary compound altered more, and tended to increase with increase in pH. From comparison of the \( \log(V_{\text{max}}/K_m) \) versus pH plots for both phthalazine and N-methyl-2-phenylquinolinium perchlorate, it would appear that binding of either substrate involves a group (or groups) on the enzyme with \( pK \) in the region of 7.7-7.9. From Figure 4.17, the greatest difference in activity between these two substrates with rabbit aldehyde oxidase occurs at the extremes of the pH range investigated.

Inhibition studies carried out at pH 7 suggest that both non-quaternary and quaternary compounds act at the same site on the enzyme. Further inhibition studies were performed at pH 6.4 and 9.3 to determine whether variation of activity with pH of these two types of substrate was due to ionisation changes occurring at the same active site. The results of this study are reported in the following section.

### 4.6.2.3 Variation of \( K_z \) with pH

Inhibition of the aldehyde oxidase catalysed oxidation of phthalazine by ethidium bromide was monitored at two pH values, 6.4 and 9.3 using rabbit enzyme. The results are shown in Figures 4.18a and b. These \( 1/v \) against \( 1/s \) plots clearly demonstrate the competitive nature of the inhibition by the N-heterocyclic cation, ethidium bromide, at both pH values. The \( K_z \) values calculated from these results were:

- \( \text{pH 6.4} \quad K_z = 2.9 \times 10^{-6} \text{ M} \)
- \( \text{pH 9.3} \quad K_z = 4.4 \times 10^{-7} \text{ M} \)
Figure 4.17. Comparison of the influence of pH on \( \log\left(\frac{V_{\text{max}}}{K_m}\right) \) values for phthalazine and N-methyl-2-phenylquinolinium.
Figure 4.18. Inhibition by ethidium bromide of the rabbit aldehyde oxidase catalysed oxidation of phthalazine at

(a) pH 6.4

Oxidation of phthalazine (△) and in the presence of 3.9 × 10⁻⁶ M (▲), 6.5 × 10⁻⁶ M (□) and 1.3 × 10⁻⁵ M (▲) ethidium bromide.

(b) pH 9.3

Oxidation of phthalazine (▼) and in the presence of 6.5 × 10⁻⁷ M ethidium bromide (▼)
With increase in pH, the $K_z$ value obtained for ethidium bromide decreased, i.e. it became a better inhibitor. The pH dependence of $K_z$ for this cation is similar to the pH dependence observed for $K_m$ for heteroaromatic cations as substrates.

Inhibition of the oxidation of N-methylquinolinium perchlorate by ethidium bromide was also monitored at the same pH values, i.e. 6.4 and 9.3. The results are shown in Figures 4.19a and b. These $1/v$ against $1/s$ plots clearly demonstrate the competitive nature of the inhibition by ethidium bromide at both pH values. The $K_z$ values calculated from these results were:

$$
\begin{align*}
\text{pH 6.4} & = 6.8 \times 10^{-6} \text{ M} \\
\text{pH 9.3} & = 5.7 \times 10^{-7} \text{ M}
\end{align*}
$$

The competitive inhibition of the oxidation of phthalazine by ethidium bromide at pH 6.4 and 9.3 suggests that at both pH values, these two compounds are acting at the same active site. The $K_z$ values obtained for ethidium bromide using either phthalazine or N-methylquinolinium are in reasonable agreement and thus provides further evidence for a common site on the enzyme capable of binding both quaternary and non-quaternary compounds.

4.6.2.4 pH profiles for the oxidaton of N-methylquinolinium perchlorate

Differences have been observed between guinea pig and rabbit liver aldehyde oxidases with regard to their interaction with N-heterocyclic cations, implying that these two enzymes differ at their active sites. Therefore it would be interesting to compare pH profiles for the oxidation of a N-heterocyclic compound with rabbit and guinea pig enzyme. N-Methylquinolinium perchlorate was chosen because it is oxidised at a reasonable rate with both enzymes. This compound does not undergo any change in ionisation over the pH range studied.
Figure 4.19. Inhibition by ethidium bromide of the rabbit aldehyde oxidase catalysed oxidation of N-methylquinolinium

(a) at pH 6.4

Oxidation of N-methylquinolinium (△) and in the presence of $1.3 \times 10^{-5}$ M (▲) and $2.6 \times 10^{-5}$ M (□) ethidium bromide

(b) at pH 9.4

Oxidation of N-methylquinolinium (▲) and in the presence of $1.0 \times 10^{-6}$ M ethidium bromide
(i) With rabbit liver aldehyde oxidase

Table 4.7. Variation of $K_m$ and $V_{\text{max}}$ values for N-methylquinolinium perchlorate with pH

<table>
<thead>
<tr>
<th>pH</th>
<th>$K_m$ a/M</th>
<th>$V_{\text{max}}$</th>
<th>log($V_{\text{max}}$/$K_m$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.48</td>
<td>2.66 x 10^{-3} (0.994)</td>
<td>0.029</td>
<td>1.04</td>
</tr>
<tr>
<td>5.97</td>
<td>2.59 x 10^{-3} (0.994)</td>
<td>0.059</td>
<td>1.36</td>
</tr>
<tr>
<td>6.52</td>
<td>1.88 x 10^{-3} (0.996)</td>
<td>0.100</td>
<td>1.72</td>
</tr>
<tr>
<td>7.02</td>
<td>1.56 x 10^{-3} (0.999)</td>
<td>0.115</td>
<td>1.87</td>
</tr>
<tr>
<td>7.89</td>
<td>4.95 x 10^{-4} (0.997)</td>
<td>0.113</td>
<td>2.36</td>
</tr>
<tr>
<td>8.72</td>
<td>3.06 x 10^{-4} (0.995)</td>
<td>0.127</td>
<td>2.61</td>
</tr>
<tr>
<td>9.03</td>
<td>5.36 x 10^{-4} (0.997)</td>
<td>0.206</td>
<td>2.58</td>
</tr>
<tr>
<td>9.39</td>
<td>7.00 x 10^{-4} (0.993)</td>
<td>0.249</td>
<td>2.55</td>
</tr>
<tr>
<td>9.94</td>
<td>5.43 x 10^{-4} (0.996)</td>
<td>0.234</td>
<td>2.60</td>
</tr>
<tr>
<td>10.20</td>
<td>4.64 x 10^{-4} (0.995)</td>
<td>0.183</td>
<td>2.60</td>
</tr>
</tbody>
</table>

a Figures in parentheses are correlation values
b Units are pmol substrate/min/mg

The $K_m$ values for N-methylquinolinium perchlorate with rabbit liver aldehyde oxidase did not alter very much over the pH range investigated. From the $V_{\text{max}}$ values maximal activity appears to occur at ca. pH 9.4. A log($V_{\text{max}}$/$K_m$) versus pH graph was plotted from the results in Table 4.7. As seen in Figure 4.20, from pH 10.2 to 8.7 the values of log($V_{\text{max}}$/$K_m$) remained fairly constant but decreased sharply at lower pH values. From this plot, it would appear that rabbit liver aldehyde oxidase has a group(s) at its active site with a pK ~8.1 which is important for binding N-methylquinolinium. However the tangent drawn to the curve to arrive at this value, which in theory should have integral slope, only had a slope of 0.6.
Figure 4.20. Plot of $\log(V_{max}/K_m)$ versus pH for N-methylquinolinium with rabbit aldehyde oxidase.
(ii) With guinea pig liver aldehyde oxidase

<table>
<thead>
<tr>
<th>pH</th>
<th>$K_m^a$/M</th>
<th>$V_{max}^b$</th>
<th>$\log(\frac{V_{max}}{K_m})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.30</td>
<td>$9.94 \times 10^{-4}$ (0.999)</td>
<td>0.100</td>
<td>2.00</td>
</tr>
<tr>
<td>6.60</td>
<td>$5.11 \times 10^{-4}$ (0.999)</td>
<td>0.121</td>
<td>2.37</td>
</tr>
<tr>
<td>7.05</td>
<td>$3.55 \times 10^{-4}$ (0.995)</td>
<td>0.149</td>
<td>2.62</td>
</tr>
<tr>
<td>7.50</td>
<td>$1.28 \times 10^{-4}$ (0.997)</td>
<td>0.140</td>
<td>3.04</td>
</tr>
<tr>
<td>7.90</td>
<td>$8.79 \times 10^{-5}$ (0.997)</td>
<td>0.133</td>
<td>3.17</td>
</tr>
<tr>
<td>8.40</td>
<td>$7.06 \times 10^{-5}$ (0.999)</td>
<td>0.145</td>
<td>3.31</td>
</tr>
<tr>
<td>8.95</td>
<td>$7.74 \times 10^{-5}$ (0.998)</td>
<td>0.193</td>
<td>3.40</td>
</tr>
<tr>
<td>9.60</td>
<td>$5.74 \times 10^{-5}$ (0.993)</td>
<td>0.174</td>
<td>3.48</td>
</tr>
</tbody>
</table>

$^a$ Figures in parentheses are correlation values
$^b$ Units are µmol substrate/min/mg

A seventeen fold decrease in $K_m$ was observed with increase in pH from 6.3 to 9.6 for N-methylquinolinium perchlorate with guinea pig liver aldehyde oxidase. In comparison, the change in $V_{max}$ values was not very great. The $\log(\frac{V_{max}}{K_m})$ versus pH plot of these results is shown in Figure 4.21 (the tangent to the curve has slope $\approx 1.15$), from which it would appear that guinea pig liver aldehyde oxidase has a group(s) at its active site with a pK $\approx 8$ which is important for binding N-methylquinolinium.

There were no dramatic differences observed between rabbit enzyme and guinea pig enzyme with respect to the kinetic data obtained for the oxidation of N-methylquinolinium over the pH range studied. The $K_m$ values for this compound tended to decrease with increase in pH with both sources of enzyme, although more markedly with guinea pig enzyme.
Figure 4.21. Plot of $\log(V_{\text{max}}/K_m)$ versus pH for $N$-methylquinolinium with guinea pig aldehyde oxidase.
With rabbit enzyme $V_{\text{max}}$ values were found to alter more but with both sources of the enzyme maximal activity occurred at high pH (≈9-9.5).

Aldehyde oxidase catalysed oxidation of N-methylquinolinium produces both N-methyl-2-quinolone and N-methyl-4-quinolone simultaneously. At pH 7 the ratio of 2:4 quinolone formation with rabbit enzyme is 17 and with guinea pig enzyme is 10; in addition, a small variation of the ratio with pH has been observed with rabbit enzyme (section 4.5). These factors could complicate analysis of the effect of pH on the oxidation of N-methylquinolinium. Despite this, the $\log(V_{\text{max}}/K_m)$ versus pH curves obtained for this compound with guinea pig enzyme and rabbit enzyme were very similar (cf Figures 4.20 and 4.21), both curves indicating the presence of a group(s) on the free enzyme concerned with binding N-methylquinolinium with $pK$ in the region ca. 8.

This may suggest that both rabbit and guinea pig aldehyde oxidases have similar catalytically important ionisable groups at their active centres. It must be pointed out, that some groups which do not ionise at all may be responsible for, or involved in, the interaction with the substrate, and no indication of their presence will be given by these $\log(V_{\text{max}}/K_m)$ versus pH graphs. Therefore since pH affects the oxidation of the N-heterocyclic cation, N-methylquinolinium, similarly with both sources of enzyme, the differences observed with respect to their interaction generally with this group of compounds (e.g. N-methyl-4-phenylquinolinium is a substrate for rabbit enzyme but an inhibitor of guinea pig enzyme) may be attributed to differences in non-ionisable groups at the active centres.

A further observation from these pH studies, concerning rabbit liver aldehyde oxidase, is that the $\log(V_{\text{max}}/K_m)$ versus pH curve obtained for N-methyl-2-phenylquinolinium perchlorate (which is oxidised exclusively
at position 4) is very similar to that obtained for N-methylquinolininium, the major oxidation product of which being the 2-quinolone (cf. Figures 4.16 and 4.20). Both curves indicate the presence of a group(s) on the free enzyme important for binding each compound with pK in the same region. This suggests that the same ionisable group at the active centre of the enzyme is responsible for binding both N-methylquinolininium and N-methyl-2-phenylquinolininium.

4.6.2.5 Critical analysis of the pH studies

pH studies, while often very useful, can easily lead to erroneous conclusions and should be interpreted with caution, particularly in the case of an enzyme as complex as aldehyde oxidase.

(i) Change in activity with pH may result from an effect of pH on the stability of the enzyme. Thus if the enzyme were kept at the pH under consideration for the duration of the $K_m$ determination, this may result (particularly at the extremes of the pH range studied) in gradual destruction of the enzyme and consequently spurious results. The initial velocities were therefore measured immediately after the enzyme was brought to the pH of testing, minimising the effect of denaturation.

Working with xanthine oxidase, Greenlee and Handler reported the presence of a group with a pK of 10.7 (suggested to be an amino group) at the active site, important for binding both quaternary and non-quaternary compounds. Recently Bunting et al. disputed the figure of 10.7; they pointed out that this value relies solely on the reliability of a single data point at pH 11 for both substrates and that this pH is relatively close to the region (pH >11.2) at which rapid inactivation of the enzyme occurs. If one ignores this data point, then log $K_m$ is strictly linear over the pH range 9.4-10.7 (note that a bend in the curve indicates a pK). These workers proposed the pK of the ionising group to be >11. This example was given for three reasons:
(a) it is concerned with an enzyme closely related to aldehyde oxidase;
(b) indicates that extreme caution is necessary in interpreting such data; and
(c) emphasises the problem of working at extremes of pH.

In the present study no kinetics were carried out above pH 10.2 and the pK of a catalytically important group on the enzyme was found to be \( \approx 8 \), i.e. in a pH region where one would expect minimum interference from denaturation problems.

(ii) Enzyme activity can vary with the type of buffer used.\(^{21,31}\) Two buffer systems were employed in the pH studies, Sörensen's phosphate (pH 5.5-8.2) and Sörensen's glycine II (pH 8.4-10.2). To eliminate the possibility that the observed activity change was due to change over of buffer systems used, controls were performed. Using Gomori's tris buffer (pH 7.2-9.0) which spans the buffer change-over region, \( V_{\text{max}} \) and \( K_m \) values were re-evaluated for N-methylquinolinium at pH 7.2, 8 and 8.7 and found to be very similar to those previously obtained using the two buffer systems and the data fitted on the \( \log(V_{\text{max}}/K_m) \) curve, thus validating the results with the buffer systems employed.

(iii) The pK of the free enzyme is obtained by drawing tangents to the \( \log(V_{\text{max}}/K_m) \) versus pH curve. The tangent to the sloping part of the curve should theoretically have integral slope; however in this present investigation this was not always found to be the case. A number of N-heterocyclic cations were previously studied with xanthine oxidase at alkaline pH\(^{239}\) and variation in the slope of the \( \log(V_{\text{max}}/K_m) \) versus pH plots from 0.51-1.17 was considered by these workers to be too large to be attributed to deviations from unit slope caused by experimental error. Rather, it was suggested that these variations in slope are an expression of the complex kinetic system under study, with the pH dependence of no single
rate constant being a controlling factor in most cases. The problems of analysing kinetic data from a two substrate reaction (i.e. oxidant + reductant \( \rightarrow \) reduced product + oxidised product) have been discussed by Engel. 235

The pH studies give ionisable constants of groups at the active surface of the enzyme and thus should throw valuable light on the chemical nature of these groups. However it must not be overlooked that if a group in the enzyme has a p\( K \) of the same value as that of a known chemical group, this does not prove its identity with the said group, since other groups may have similar p\( K \) values. Furthermore a given group may not have the same p\( K \) when it is present in a protein molecule, owing to the influence of the environment created by adjacent groups in the folded protein. Such shifts in p\( K \) values are, however, usually relatively small and seldom greater than 2 pH units.

In this present study, it was found that regardless of substrate used or source of enzyme (guinea pig or rabbit) aldehyde oxidase possesses a catalytically important group with a p\( K \) \( \sim \) 8 (error in this type of determination is usually ±0.3 units). This value is comparable with the values for the groups shown below. 240

\[ \begin{array}{|c|c|}
\hline
\text{Group} & \text{pK range (25\(^\circ\))} \\
\hline
\text{Sulphydryl} & 8.3-8.6 \\
\text{Ammonium (a)} & 7.6-8.4 \\
\text{Ammonium (a, cystine)} & 6.5-8.5 \\
\hline
\end{array} \]

It must be pointed out that since the pH profiles were carried out above pH 5.5, other catalytically important ionisable groups may be possessed by
aldehyde oxidase with pK lower than 5.5 which would not have been detected (e.g. pK's of carboxyl groups in proteins are generally lower than 5). Finally it should be noted that the identification of a specific ionising group from such studies should not be taken as absolute proof that the group is at the active site. It is possible that a pH-dependent reversible conformational change that led to a change in activity would give results that were indistinguishable from those due to ionisation of an essential group at the active site.

A critical analysis of the problems involved in the interpretation of the effects of pH on enzyme activity has been presented by Knowles. 241

4.7 Discussion of the Substrate Binding Site

The binding site investigations yielded a number of interesting observations which are summarised and discussed below.

Site(s) of 2-quinolone and 4-quinolone formation

Kinetic studies to determine whether 2- and 4-quinolone formation occurred at the same site on the enzyme involved monitoring the oxidation of a substrate in the presence of another compound and observing the type of inhibition exhibited. The rabbit liver aldehyde oxidase catalysed oxidation of N-methyl-4-phenylquinolinium, which yields exclusively the 2-quinolone, is competitively inhibited by N-methyl-2-phenylquinolinium (which is oxidised at position 4). This suggests that 2- and 4-quinolone formation occurs at a common site on the enzyme. The same conclusion can be drawn for guinea pig enzyme from the results in Table 4.9. These show that the oxidation of 6-methylpurine is competitively inhibited by N-heterocyclic cations regardless of whether these cations are oxidised at the 2 or 4 position.
Table 4.9. Inhibition of 6-methylpurine oxidation by N-heterocyclic cations

<table>
<thead>
<tr>
<th>Substrate Monitored</th>
<th>&quot;Inhibiting&quot; Species</th>
<th>Type of Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-Methylpurine</td>
<td>N-Methyl-4-phenylquinolinium&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Competitive</td>
</tr>
<tr>
<td>6-Methylpurine</td>
<td>N-Methyl-2-phenylquinolinium&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Competitive</td>
</tr>
<tr>
<td>6-Methylpurine</td>
<td>N-Phenyl-2-phenylquinolinium&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Competitive</td>
</tr>
</tbody>
</table>

<sup>a</sup> Very slowly oxidised to the 2-quinolone
<sup>b</sup> Very slowly oxidised to the 4-quinolone
<sup>c</sup> Guinea pig enzyme used

Furthermore, the pH profiles of N-methyl-2-phenylquinolinium and N-methyl-quinolinium (which is oxidised mainly at the 2 position) were very similar and indicated (with the provisos made in section 4.6.2.5) the presence of a group(s) on the free enzyme important for binding each compound with a pK in the same region, ca. 8. This suggests that the same ionisable group at the active centre of the enzyme is of importance with respect to the binding of both compounds.

Certain quinolinium cations (e.g. N-phenylquinolinium) have been shown to be oxidised simultaneously to 2- and 4-quinolones with aldehyde oxidase. From the results above, it can be postulated that different orientation of the substrate molecule at a single active site on the enzyme accounts for formation of the two isomeric products. Since aldehyde oxidase will oxidise a large number of nitrogeneous compounds of widely differing structures, it would not be too surprising to find that it could bind N-heterocyclic cations in two alternate configurations, each leading to oxidation at a different position. Consistent with this hypothesis, is the large size of the enzyme active site suggested above (section 4.3). Furthermore Bray et al.<sup>242</sup> using e.p.r., showed xanthine to form two types of complex with xanthine oxidase; these correspond to two different
orientations of the substrate in the active site. One such complex was said to be non-productive while the other allows catalysis. The lower ratio of 2- to 4-quinolone production from quinolinium cations with guinea pig enzyme compared with rabbit enzyme could be explained within this "one site - two orientations" hypothesis, by assuming that the active sites of the two enzymes differ somewhat. This assumption is supported by the differences observed between these enzymes with respect to their interaction with a large number of compounds, e.g. N-phenyl-2-phenyl-quinolinium is bound by both enzymes but is only turned over to any appreciable extent by rabbit enzyme. Felsted and Chaykin\textsuperscript{39} studied the oxidation of N\textsuperscript{1}-methylnicotinamide with hog, mouse and rabbit enzyme and similarly concluded that the varying amounts of 4-pyridone relative to 2-pyridone formed reflect species differences in the amino acid contents of the three enzymes and dissimilar active sites.

Scazzochio and Sealy-Lewis\textsuperscript{141,243} have considered the geometry of the active site insofar as it might explain differences in substrate-inhibitor specificities. They proposed that binding sites, by orientating the substrate in a specific way(s) in relation to the catalytically important groups, determine which carbon, if any, is to be hydroxylated. Differences between one enzyme and another would therefore reflect differences in the relative positions of the binding and catalytic groups at the active site.

In this present study, the ratio of 2- and 4-quinolone formation from N-methylquinolinium with rabbit enzyme was found to be constant under a variety of conditions (section 4.5). However the ratio was found to increase in the presence of the competing substrate N-methyl-phenanthridinium. It is difficult to envisage how this compound could inhibit formation of 4-quinolone more effectively than that of the 2-quinolone if, as the kinetic studies suggest, formation of both quinolones occurs at the same site on the enzyme. One possible explanation may be that this
particular compound, i.e. N-methylphenanthridinium (or its oxidation product) may at the concentrations used, cause conformational changes at the active site which could affect the ability of N-methylquinolinium to bind in a particular orientation.

One other possible explanation for the kinetic data and quinolone ratio results may arise from the presence of isoenzymes, one of which producing the 2-quinolone, the other the 4-quinolone. However, these isoenzymes would have to be extremely similar in their ability to "bind" substrates to explain the kinetic results, for example the 2-quinolone-yielding isoenzyme would also have to bind N-methyl-2-phenylquinolinium to account for the competitive nature of inhibition observed between this substrate and N-methyl-4-phenylquinolinium. Also since phthalazine-induced aldehyde oxidase gave the same 2- to 4-quinolone ratio as was observed with uninduced enzyme, then both isoenzymes would have to be similarly induced. On the other hand, it is easier to visualise, using the two isoenzyme concept, the differential inhibitory action of N-methylphenanthridinium towards 2- and 4-quinolone formation and also, if mammals possessed different levels of the 2 isoenzymes then this would account for the different 2:4-quinolone ratios observed between the species. The presence of the two isoenzyme forms of aldehyde oxidase in mouse has been demonstrated by Holmes using electrophoretic techniques, although the only substrate monitored with both isoenzymes was benzaldehyde. The most abundant isoenzyme form was located in the liver and pancreas along with lower levels of a second form; low levels of this latter form were also found in other tissues, e.g. kidney and stomach. In view of this work, it would be very interesting to compare 2- to 4-quinolone ratios obtained using aldehyde oxidase prepared from mouse stomach (i.e. one isoenzyme) and mouse liver (i.e. with both isoenzymes).

Recently, two forms of what appears to be aldehyde oxidase have been
isolated from rat.\textsuperscript{48} N-methylnicotinamide is oxidised by one of the forms (oxidase I) to yield both 2- and 4-pyridone but interestingly the other form (oxidase II) only yields the 4-pyridone. However this work has only been carried out with rat, a mammal which is known to exhibit anomalous behaviour with respect to N\textsuperscript{1}-methylnicotinamide oxidation (as discussed in section 1.2.2). Moreover, the oxidase II was found to be specific towards N\textsuperscript{1}-methylnicotinamide, although quinolinium cations were not among the compounds tested as substrates.

Preliminary observations in this laboratory using isoelectric focusing techniques have shown that purified rabbit liver aldehyde oxidase gives rise to 2 bands of activity in close juxtaposition.\textsuperscript{233}

The question, as to whether isoenzymes are responsible for the variation of quinolone ratio in rabbit and guinea pig enzyme, can only be resolved if the two forms can be isolated in sufficient quantity and tested independently.

\textit{Site(s) of interaction of quaternary and non-quaternary compounds}

Oxidation of the non-quaternary compound, 6-methylpurine is competitively inhibited by quinolinium compounds using guinea pig liver enzyme as shown in Table 4.9. These results suggest that quaternary and non-quaternary compounds act at a common site on the enzyme. The same conclusion can be drawn from the results in Table 4.10 for rabbit enzyme.

\begin{table}
\centering
\begin{tabular}{lll}
\hline
Substrate monitored& "Inhibiting" species & Type of Inhibition observed \\
\hline
6-methylpurine & N-methylisoquinolinium & competitive \\
6-methylpurine & N-methyl-4-phenylquinolinium & competitive \\
6-methylpurine & ethidium bromide & competitive \\
phthalazine (pH 6.4) & ethidium bromide & competitive \\
phthalazine (pH 9.3) & ethidium bromide & competitive \\
\hline
\end{tabular}
\caption{Inhibition of the rabbit enzyme catalysed oxidation of non-quaternary compounds by N-heterocyclic cations}
\end{table}

\textsuperscript{a} pH 7 unless otherwise stated
Rajagopalan and Handler\textsuperscript{3} found quinacrine to be a competitive inhibitor of aldehyde oxidase with either \( N^1 \)-methyl nicotinamide or salicylaldehyde as substrate and the \( K_z \) values obtained to be essentially the same in both cases. However, they also found that whereas the irreversible inactivation of the enzyme by cyanide was significantly retarded by quinacrine and uncharged substrates, this was not the case for substrates bearing a positive charge. The inhibitory action of cyanide is considered to be due to its abstraction of an essential sulphur atom from the molybdenum centre. Therefore although evidence points to quaternary and non-quaternary compounds acting at the same site on the enzyme, it would appear that subtle differences must exist with respect to the interaction of these two types of compound with the cyanolysable sulphur.

In this present study, rabbit enzyme was found to be active towards both phthalazine and \( N \)-heterocyclic cations, over a wide pH range (5.5-10). With increase in pH the \( K_m \) values for the non-quaternary compound increased whilst those for the cations decreased. From graphical analysis of the data, it appeared (with the provisos made in section 4.6.2.5) that binding of either class of substrate involved a group (or groups) on the enzyme with \( pK \) in the region ca. 8. Oxidation of phthalazine was found to be competitively inhibited by an \( N \)-heterocyclic cation (ethidium bromide) at pH 6.4 and 9.3. This suggests that the variation of activity with pH of quaternary and non-quaternary substrates is due to ionisation changes of the same group(s) at the active site of the enzyme. Thus in the protonated form, the group may prevent binding of an \( N \)-heterocyclic cation by electrostatic repulsion and thus account for the increase in \( K_m \) observed at lower pH values; in contrast, the binding of the uncharged phthalazine molecule is facilitated at these lower pH's.
This could be represented:

**Figure 4.22.** Schematic representation of the interaction of quaternary and non-quaternary substrates with an ionisable group (X) on the enzyme.

pH < 8

\[ \text{X-H} \quad \text{ELECTROSTATIC REPULSION} \quad \text{H}_2\text{C-N}^+ \]

\[ \text{X-H} \quad \text{HYDROGEN BONDING} \quad \text{CN} \]

pH > 8

\[ \text{X} \quad \text{ELECTROSTATIC ATTRACTION} \quad \text{H}_2\text{C-N}^+ \]

\[ \text{X} \quad \text{ELECTROSTATIC REPULSION} \quad \text{CN} \]

Other ionisable groups with pKs outside the pH range studied may also contribute to binding (*e.g.* -COOH).

Interaction of N-heterocycles with molybdenum hydroxylases is generally considered to involve initial binding of the substrate via nitrogen to the molybdenum atom of the enzyme. However this proposal and subsequent mechanisms of hydroxylations have been based on non-quaternary compounds, *e.g.* xanthine.
It is difficult to envisage how quaternary compounds, which do not possess a lone pair of electrons on nitrogen, bind in this fashion. In addition, N-heterocyclic cations which possess extremely sterically hindered nitrogen atoms have also been found to interact with aldehyde oxidase, e.g. N-phenyl-2-phenylquinolinium is one of the best substrates known for rabbit enzyme.

Although the reducing substrate binding site has been established as the molybdenum centre,¹⁴³ no firm evidence has so far been presented to support substrates binding directly to the molybdenum atom, which would require the metal to have a vacant coordination site. Since it is thought that both quaternary and non-quaternary compounds act at a common site on xanthine oxidase,³³ and on aldehyde oxidase (see above), then it could be proposed that neither class of substrate binds via nitrogen directly to molybdenum. The question then arises, how do these substrates bind to the enzyme?

From the results presented for aldehyde oxidase, it is reasonable to suggest that ionisable groups (see Figure 4.22) and/or hydrophobic interactions (section 4.3) at the enzyme active site are important for substrate binding. This type of "loose" interaction may allow different orientations of the substrate at the active centre and thus account for the simultaneous production of 2- and 4-quinolones.
Whilst this work was in progress, a hydroxylation mechanism was proposed by Davies (Ph.D. Thesis, Rice University) and although it was for xanthine oxidase, the basic ideas are similar to those suggested above for aldehyde oxidase. For example, molybdenum was not shown to be involved in the initial enzyme-substrate complex (Figure 4.23, where the example of the substrate shown is 2,4-dihydroxypteridine). This hypothesis was based on data which indicated that there is little or no change in the optical properties of either the enzyme or substrate during the rapid equilibrium for the 'enzyme - 2,4-dihydroxypteridine' binding step. Instead the enzyme-substrate interaction is shown to be a function of hydrogen bonding; this proposal arises from the observation that the dissociation constant for the enzyme-substrate complex alters dramatically with pH. It is a subsequent conformational change of the enzyme which is thought to bring bound 2,4-dihydroxypteridine into the coordination sphere of the molybdenum. The interaction of the oxo-ligand of the molybdenum was then postulated to induce covalent hydration to occur across the 7,8-double bond. The C-7 proton and two electrons are abstracted in the form of a hydride ion by the sulphido-ligand of the molybdenum; the metal being reduced from Mo(VI) to Mo(IV). Finally the product is released (in this example 2,4,7-trihydroxypteridine) and the enzyme is reoxidised.

Any mechanism of action proposed for aldehyde oxidase must take into account the following observations made in this thesis:

(i) Quinolinium compounds in which the ring nitrogen is sterically hindered can act as substrates for aldehyde oxidase.

(ii) Oxidation of quinolinium compounds can occur at a site removed from, as well as adjacent to, the ring nitrogen. 4-Quinolones can be formed either simultaneously, along with the 2-quinolones, or
Figure 4.23. Initial binding steps in the mechanism of xanthine oxidase catalysed oxidation of 2,4-dihydroxypteridine, as proposed by Davis.\textsuperscript{228}
exclusively, from cations with a blocked 2-position.

(iii) From kinetic studies, it is apparent that quaternary and non-quaternary compounds interact at the same site on the enzyme.

(iv) With increase in pH, $K_m$ values for compounds bearing a positive nitrogen decrease whilst the $K_m$ values for non-quaternary substrates increase.

No currently accepted mechanism adequately explains all these results.
CHAPTER 5

Pseudobases as Substrates for Aldehyde Oxidase

5.1 Introduction

In 1899, Hantzch introduced the term "pseudobase" to describe the covalent hydroxide adducts of quaternary nitrogen heteroaromatic cations. Such adducts are cyclic carbinolamines if hydroxide ion addition occurs at the carbon atom adjacent to the quaternary nitrogen atom, e.g. (1), or simply carbinols if addition occurs at a position that is remote from the nitrogen atom, e.g. (2).

For the equilibrium between the quaternary heterocyclic cation \( Q^+ \) and the corresponding pseudobase \( QOH \),

\[
Q^+ + OH^- \rightleftharpoons QOH
\]

an association equilibrium constant is given by equation (1).

\[
K = \frac{[QOH]}{[Q^+][OH^-]} \quad (1)
\]
Alternatively this equilibrium may be expressed as:

\[ \text{Q}^+ + \text{H}_2\text{O} \rightleftharpoons \text{QOH} + \text{H}^+ \]

with an equilibrium constant \( K_{\text{ROH}} \) (equation (2)) which has the form of a classical Brønsted acid ionisation constant.

\[
K_{\text{ROH}} = \frac{[\text{H}^+][\text{QOH}]}{[\text{Q}^+]}
\]

It is convenient to express these equilibrium constants as \( pK_{\text{ROH}} \) values which are analogous to \( pK_{\alpha} \) values for Brønsted acids, and which denote the pH at which the quaternary ion and pseudobase are present in equal concentrations.\(^{147}\)

Pseudobases have been postulated as intermediates in many reactions of N-heterocyclic cations,\(^{145}\) \( e.g. \) bromination\(^{245}\) and disproportionation.\(^{246}\) It is the involvement of pseudobases in chemical oxidation (see below) which is of particular interest in the current studies since aldehyde oxidase catalysed oxidation, which is considered to be a nucleophilic process, may involve a pseudobase or "pseudobase-type" \( (e.g. \) R–S\(^-\) attack) intermediate. It would thus be interesting to investigate whether a compound which was essentially in the pseudobase form at pH 7 is a substrate for aldehyde oxidase.

![Chemical structures](attachment:image.png)
The $pK_{ROH}$ values for N-methyl quinolinium, isoquinolinium and benzoquinolinium cations have been previously reported and are all $>10$ (see Table 5.1). Upon pseudobase formation, dramatic spectral changes occur since nucleophilic addition to an unsaturated carbon atom leads to significant changes in the electronic configuration of the molecule, particularly if pseudobase formation disrupts the aromatic character of ring. The u.v. spectra of all other N-heterocyclic cations used thus far in this thesis were recorded at pH 1, pH 7 and pH 10 and found to be essentially identical. Therefore the N-heterocyclic cations studied in Chapters 3 and 4 exist exclusively in the ionic form.

Despite pseudobase formation being investigated since the turn of the century, it is only recently that attempts to correlate $pK_{ROH}$ and cation structure have been made, $^{147,247}$ the key factors of which are briefly discussed below:

(i) Loss in resonance energy militates against pseudobase formation. For example, N-methyl-3,4-dihydroisoquinolinium has a $pK_{ROH}$ value 4.5 units smaller than that of its aromatic analogue N-methylisoquinolinium; the greater reluctance of the latter cation towards pseudobase formation results from the enhanced stability of the $C-N^+$ bond via resonance.

(ii) Introduction of electron-withdrawing substituents destabilises the cation relative to the pseudobase and results in the lowering of $pK_{ROH}$ relative to the parent cation. A striking example of this effect is that the $pK_{ROH}$ value for N-cyanoquinolinium is ca. 17.5 units lower than that for the N-methylquinolinium cation. Similarly introduction of additional ring nitrogen atoms results in a reduction of $pK_{ROH}$ (e.g. N-methylquinolinium ca. 16.5, $N^1$-methylquinoxalinium 8.62)
Table 5.1. \( pK_{ROH} \) values for N-heterocyclic cations

<table>
<thead>
<tr>
<th>Cation</th>
<th>( pK_{ROH} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-methylquinolinium</td>
<td>( \sim 16.5 )</td>
</tr>
<tr>
<td>N-cyanoquinolinium</td>
<td>-0.86, -1.05</td>
</tr>
<tr>
<td>N-methylisoquinolinium</td>
<td>( \sim 15.3, 16.29 )</td>
</tr>
<tr>
<td>N-cyanoisoquinolinium</td>
<td>-2.10</td>
</tr>
<tr>
<td>N-methyl-3,4-dihydroquinolinium</td>
<td>10.75</td>
</tr>
<tr>
<td>N-methylphenanthridinium</td>
<td>10.4, 11.94</td>
</tr>
<tr>
<td>N-methyl-5,6-benzoquinolinium</td>
<td>&gt;14</td>
</tr>
<tr>
<td>N-methyl-7,8-benzoquinolinium</td>
<td>&gt;14</td>
</tr>
<tr>
<td>N2-methylphthalazinium</td>
<td>11.04</td>
</tr>
<tr>
<td>N1-methylquinoxalinium</td>
<td>8.62</td>
</tr>
<tr>
<td>N3-methylquinazolinium</td>
<td>&lt;7</td>
</tr>
</tbody>
</table>

A problem encountered in the study of pseudobases, is the possibility of ring-chain tautomerism between pseudobase carbinolamines and their amino-carbonyl isomers:

\[
\begin{array}{ccc}
\text{N} & \text{R} & \text{OH}^- \\
\text{N} & \text{R} & \text{OH} \\
\text{NH} & \text{R} & \text{CO}
\end{array}
\]

However the pseudobases derived from quinolinium and isoquinolinium cations are much less susceptible to ring opening than their pyridinium analogues. Furthermore recent examples in which the ring-opened species
have been characterised beyond doubt are confined to strongly electron
withdrawing substituents on the ring nitrogen atom.

5.2 Characterisation of a Pseudobase

Only a few N-heterocyclic cations have been reported to be in the
terbase form at pH 7. Interestingly in 1979 Parg et al. found
that 3-methyl-2-oxoquinazolinium chloride (A) in a neutralised aqueous
solution gave rise to the insoluble pseudobase 3,4-dihydro-4-hydroxy-3-
methyl-2-quinazolinone (B) which was isolated and identified. This could
mean either that the pseudobase is the predominant species in the equilibrium
or simply the less soluble form. This equilibrium thus warranted further
investigation.

\[
\begin{align*}
\text{(A)} & \quad \text{NCH}_3 + \text{OH}^- \quad \rightleftharpoons \quad \text{(B)} \\
\end{align*}
\]

The pK\text{ROH} value for 3-methyl-2-oxoquinazolinium chloride (A) was
determined spectrophotometrically as described by Albert and Serjeant. This method involves determining the relative proportions of (A) to (B)
in a series of solutions of accurately known pH values. This is done at
a wavelength (called the analytical wavelength) at which the greatest
difference in optical densities between the cation (A) and the neutral
molecule (B) is observed. The pK\text{ROH} is obtained from the equation:

\[
\text{pK}_{\text{ROH}} = \text{pH} + \log \frac{\epsilon - \epsilon_M}{\epsilon_I - \epsilon} \quad (3)
\]

Where \(\epsilon_I\) is the extinction coefficient of the ion (A) at the analytical
wavelength, $\varepsilon_M$ is the extinction coefficient of the molecule (B) at the same wavelength, and $\varepsilon$ is the extinction coefficient of the mixture of ion and molecule at the same wavelength. Changes in the value of $\varepsilon$ with variation of pH allows solution of equation (3) at various degrees of ionisation of the molecule. Provided that the same concentrations are used, equation (3) may be written with absorbances (d) replacing extinction coefficients ($\varepsilon$).

A stock solution of $1.074 \times 10^{-3}$ M 3-methyl-2-oxoquinazolinium chloride (A) was prepared in water. The u.v. spectra obtained for a 1 in 10 dilution of the stock solution at pH 0.5 and pH 0.87 were identical and attributed to the cation (A); the absorbance at 300 nm was found to be 1.272. The spectra at pH 7 and pH 13 were identical (but different from those obtained at pH 0.5 and 0.87) and hence attributed to the pseudobase (B), which was not found to absorb at 300 nm. Consequently 300 nm was selected for the analytical wavelength ($d_1 = 1.272$, $d_M = 0$).

The stock solution was then diluted 1 in 10 with Sørensen's glycine/hydrochloric acid buffer adjusted to pH 2.5. The absorbance (at 300 nm) was found to be 0.272 and the $pK_{ROH}$ calculated to be 1.93. Six more solutions were then prepared (1 in 10 dilutions of stock solution with buffer) spanning the pH range 1.20 to 2.67 and the absorbance of each at 300 nm measured (see Table 5.2). The $pK_{ROH}$ values obtained were averaged to give $1.94 \pm 0.06$; thus at pH 7 the compound may be regarded as being almost exclusively in the pseudobase form (B). Furthermore, the pseudobase (B), rather than its ring opened amino-carbonyl tautomer, was found to be the predominant species, since the u.v. spectrum of the methoxide adduct of (A) formed in basic methanol, i.e. (C) (which is incapable of ring opening) was identical to the u.v. spectrum of the pseudobase obtained at pH 7.
Figure 5.1. The pH dependence of the u.v. spectrum of 3-methyl-2-oxoquinazolinium chloride ($10^{-4}$ M)

1, pH 1.20; 2, pH 1.62; 3, pH 1.78; 4, pH 2.01; 5, pH 2.29; 6, pH 2.50; 7, pH 2.67

Table 5.2. Determination of the $pK_{ROH}$ for 3-methyl-2-oxoquinazolinium chloride ($A$)

<table>
<thead>
<tr>
<th>pH</th>
<th>$d$</th>
<th>$\log \frac{d - d_M}{d_I - d}$</th>
<th>$pK_{ROH}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.20</td>
<td>1.100</td>
<td>+0.80</td>
<td>2.00</td>
</tr>
<tr>
<td>1.62</td>
<td>0.870</td>
<td>+0.34</td>
<td>1.96</td>
</tr>
<tr>
<td>1.78</td>
<td>0.771</td>
<td>+0.19</td>
<td>1.97</td>
</tr>
<tr>
<td>2.01</td>
<td>0.568</td>
<td>-0.09</td>
<td>1.92</td>
</tr>
<tr>
<td>2.29</td>
<td>0.373</td>
<td>-0.38</td>
<td>1.91</td>
</tr>
<tr>
<td>2.50</td>
<td>0.272</td>
<td>-0.57</td>
<td>1.93</td>
</tr>
<tr>
<td>2.67</td>
<td>0.189</td>
<td>-0.76</td>
<td>1.91</td>
</tr>
</tbody>
</table>

Analytical wavelength = 300 nm
Concentration of solutions = $1.074 \times 10^{-4}$ M
Temp. = 30°C, $d_M = 0$, $d_I = 1.272$
If the pseudobase had existed in the ring opened tautomeric form, then one would have expected the u.v. spectrum to be very different from that of the methoxide adduct.²⁵¹

The 3-methylquinazolinium cation (D) is known to undergo hydroxide ion attack at C-4 to give a pseudobase and the $pK_{\text{ROH}}$ for this cation has been indirectly estimated to be ca. 5²⁵² (see section 5.5).
The $pK_{\text{ROH}}$ value for the cation (A) would be expected to be lower than 5 since:

(i) cation (A) possesses an electron withdrawing group, and
(ii) addition of a hydroxide ion disrupts the aromaticity of the cation (D).

Thus the $pK_{\text{ROH}}$ value obtained for cation (A) of 1.94 would appear reasonable. Thus it has been established with a reasonable degree of certainty that at pH 7 3-methyl-2-oxoquinazolinium (A) exists as the pseudobase (B). This uncharged compound (B) was isolated and found to be stable to aerial oxidation (i.e. it can be recrystallised in air from boiling solvents such as dioxan).

5.3 **Incubation of 3,4-Dihydro-4-hydroxy-3-methyl-2-quinazolinone with Aldehyde Oxidase**

Addition of aldehyde oxidase (from rabbit or guinea pig) to the pseudobase (B) at pH 7, resulted in a change in the u.v. spectrum (see Figure 5.2). When this procedure was repeated in the presence of the potent aldehyde oxidase inhibitor menadione, no such change in u.v. spectrum was observed. These findings suggest the pseudobase, 3,4-dihydro-3-methyl-2-quinazolinone (B), is a substrate for aldehyde oxidase. The u.v. of the oxidation product is the same as that recorded for 3-methyl-2,4-quinazolinedione (E) (see Figure 5.3).
Figure 5.2. The u.v. spectrum of 3,4-dihydro-4-hydroxy-3-methyl-2-quinazolinone and of the product of its oxidation catalysed by aldehyde oxidase.

(a) \(10^{-4}\) M solution of 3,4-dihydro-4-hydroxy-3-methyl-2-quinazolinone in pH 7 phosphate buffer.

(b) As (a) plus aldehyde oxidase (from guinea pig or rabbit), incubated for 30 mins at 30°C.

(c) As (b) with 2 \(x\) \(10^{-5}\) M menadione.

Figure 5.3. The u.v. spectrum of 3-methyl-2,4-quinazolinedione*
The pseudobase (B) was therefore incubated at pH 7 with partially purified preparations of either rabbit or guinea pig enzyme (incubation conditions and controls were similar to those described in section 2.4.2). After one hour methanol was added to the incubation mixture to precipitate the protein and thus terminate the reaction. An h.p.l.c. system was developed to separate the pseudobase (B) and its incubation product. 20 μl portions of the incubation mixture were injected directly on to the h.p.l.c. column.

**H.p.l.c. data**

<table>
<thead>
<tr>
<th>Retention Volumes/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P2</strong></td>
</tr>
<tr>
<td>Pseudobase (B)</td>
</tr>
<tr>
<td>Metabolite</td>
</tr>
<tr>
<td>Dione (E)</td>
</tr>
</tbody>
</table>

Conditions as in section 2.5.2, except monitored at 250 nm

* Very sharp peaks

Samples of the metabolite were collected from the h.p.l.c. eluate and the infrared and mass spectra recorded.

The mass spectra of 3-methyl-2,4-quinazolinedione (E) and the metabolite are identical (see Figure 5.4). Both show a molecular ion peak at m/e 176 and the most abundant fragment ion peak at m/e 119. In contrast, the mass spectrum for the substrate (B) has a molecular ion peak at m/e 178 and an intense peak at m/e 160 (M-H₂O).

The metabolite has an infrared spectrum which is characterised by bands in the carbonyl stretching region at 1720 and 1665 cm⁻¹ and is very similar to that of (E) (Figure 5.5). The higher frequency band is
Figure 5.4. Mass spectra of 3,4-dihydro-4-hydroxy-3-methyl-2-quinazolinone, 3-methyl-2,4-quinazolinedione and the incubation product of 3,4-dihydro-4-hydroxy-3-methyl-2-quinazolinone.
Figure 5.5. Infrared spectra of 3-methyl-2,4-quinazolinedione and the incubation product of 3,4-dihydro-4-hydroxy-3-methyl-2-quinazolinone

3-Methyl-2,4-quinazolinedione

Metabolite

Wavenumber [cm⁻¹]
attributed to the carbonyl in position 4 because of its proximity to the benzene ring and the lower frequency band to the carbonyl in position 2. The infrared spectrum of the substrate (B) has a single carbonyl band at 1660 cm$^{-1}$.

From these results it may be concluded that incubation of the pseudobase (B) yields 3-methyl-2,4-quinazolinedione and that the enzyme responsible for catalysing this dehydrogenation is aldehyde oxidase.

5.4 Kinetic Study

Rates of enzymatic oxidation of the pseudobase (B) were monitored spectrophotometrically, using oxygen as the electron acceptor, by following the increase in absorbance at 310 nm corresponding to product formation (see section 2.6.2.3). The $v$ (rate) and $s$ (substrate concentration) data was treated as outlined in section 4.2 to obtain the Michaelis-Menten constants.

(a) with rabbit liver aldehyde oxidase (see Figure 5.6a)

$$K_m = 6.1 \times 10^{-4} \text{ M} \quad (0.999)$$

(b) with guinea pig liver aldehyde oxidase (see Figure 5.6b)

$$K_m = 5.4 \times 10^{-4} \text{ M} \quad (0.999)$$

With both enzymes substrate inhibition was observed at concentrations in excess of $3 \times 10^{-3}$ M.

From the $K_m$ values and the fact that reasonable rates of oxidation were observed it can be concluded that the pseudobase (B) is quite a good substrate for aldehyde oxidase from either guinea pig or rabbit. In contrast this compound was not found to be oxidised by xanthine oxidase; however, this does not rule out the possibility that other compounds of the same class (i.e. pseudobases) might serve as substrates for this enzyme.
Figure 5.6. Determination of the Michaelis constants for 3,4-dihydro-4-hydroxy-3-methyl-2-quinazolinone.

(a) with rabbit aldehyde oxidase

(b) with guinea pig aldehyde oxidase
The effect of pH upon $K_m$ and $V_{\text{max}}$ values for pseudobase (B) with rabbit enzyme was monitored under similar conditions to those described in section 2.6.2.4, except that rates were measured at 310 nm (i.e. not in the presence of ferricyanide). The concentration of the pseudobase (B) required to observe substrate inhibition increased with increase in pH, paralleling the change in $K_m$.

Table 5.3. Variation of $K_m$ and $V_{\text{max}}$ values with pH for 3,4-dihydro-4-hydroxy-3-methyl-2-quinazolinone (B)

<table>
<thead>
<tr>
<th>pH</th>
<th>$K_m^a$/M</th>
<th>$V_{\text{max}}^b$</th>
<th>$\log(\frac{V_{\text{max}}}{K_m})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.05</td>
<td>$4.20 \times 10^{-4}$ (0.999)</td>
<td>0.044</td>
<td>2.02</td>
</tr>
<tr>
<td>6.75</td>
<td>$4.79 \times 10^{-4}$ (0.997)</td>
<td>0.074</td>
<td>2.19</td>
</tr>
<tr>
<td>7.70</td>
<td>$1.03 \times 10^{-3}$ (0.998)</td>
<td>0.0995</td>
<td>1.98</td>
</tr>
<tr>
<td>8.00</td>
<td>$2.90 \times 10^{-3}$ (0.999)</td>
<td>0.171</td>
<td>1.77</td>
</tr>
<tr>
<td>8.75</td>
<td>$5.64 \times 10^{-3}$ (0.999)</td>
<td>0.087</td>
<td>1.18</td>
</tr>
<tr>
<td>9.30</td>
<td>$6.94 \times 10^{-3}$ (0.997)</td>
<td>0.052</td>
<td>0.88</td>
</tr>
</tbody>
</table>

$^a$ Values in parentheses are correlation figures
$^b$ Units are $\mu$mol/min/mg protein calculated using an extinction coefficient of 3,500 for the product (F), which was found to be constant over the pH range investigated

A sixteen-fold increase in $K_m$ was observed with increase in pH from 6.05 to 9.3. A smaller change in $V_{\text{max}}$ of ca. 4 was observed over this pH range, with maximal activity exhibited at ca. pH 8. A $\log(\frac{V_{\text{max}}}{K_m})$ versus pH graph was plotted from the results (see Figure 5.7). It is noteworthy that the shape of the curve is similar to that obtained for the uncharged substrate phthalazine and indicates a pK value for the enzyme in the region of ca. pH 7.5. However as this curve is derived using only 6 points it would be unwise to draw any firm conclusions from it.
Figure 5.7. Plot of $\log(V_{max}/K_m)$ versus pH plot for 3,4-dihydro-4-hydroxy-3-methyl-2-quinazolinone with rabbit aldehyde oxidase.

\[ \log(V_{max}/K_m) \]

$\text{pH}$

0.8 1.0 1.2 1.4 1.6 1.8 2.0 2.2 2.4

6.0 6.5 7.0 7.5 8.0 8.5 9.0 9.5
Normally aldehyde oxidase catalysed oxidation results in hydroxylation of substrate, but oxidation of the pseudobase (B) involves only dehydrogenation. It was of interest to determine whether the pseudobase acted at the same site on the enzyme as the hydroxylatable substrates. Therefore the rates of oxidation of the pseudobase (B) were compared both in the absence and presence of 6-methylpurine (\(1.6 \times 10^{-3} \text{ M; } 2.6 \times 10^{-3} \text{ M}\)) as a competing substrate. When monitored at 310 nm, there is no contribution to the change in absorbance arising from oxidation of the latter substrate. A Lineweaver-Burk plot of these results is shown in Figure 5.8 and demonstrates quite clearly that 6-methylpurine competitively inhibits the oxidation of the pseudobase (B). The \(K_z\) value was calculated to be \(1.4 \times 10^{-3} \text{ M}\) which is in very good agreement with its \(K_m\) value of \(1.5 \times 10^{-3} \text{ M}\).

From these results it would appear that oxidation of 6-methylpurine and the pseudobase (B) occurs at the same site on the enzyme. Furthermore, the potent inhibitor ethidium bromide (a quaternary compound) which was shown in Chapter 4 to act at the substrate binding site, was also found to inhibit the oxidation of the pseudobase (B).

5.5 Preliminary Studies with the Pseudobase 3,4-Dihydro-4-hydroxy-3-methylquinazoline

To support the generalisation that pseudobases, as a class of compounds, can act as substrates for aldehyde oxidase another example was sought.

The 3-methylquinazolinium cation (D) forms a pseudobase (F) and the \(pK_{ROH}\) for this cation has been indirectly estimated to be \(ca. 5.25\).
Figure 5.8. Inhibition by the competing substrate, 6-methylpurine, of the rabbit aldehyde oxidase catalysed oxidation of 3,4-dihydro-4-hydroxy-3-methyl-2-quinazolinone.

Oxidation of 3,4-dihydro-4-hydroxy-3-methyl-2-quinazolinone (■) and in the presence of 1.6 x 10^{-3} M (□) and 2.6 x 10^{-3} M (▲) 6-methylpurine.
However pseudobase formation is complicated by its facile protonation:

\[
\text{H} \quad \text{OH} \\
\text{NCH}_3 \\
\text{F} \\
\text{H} \quad \text{OH} \\
\text{NCH}_3 \\
\text{F-H}^+ \\
\]

The \( pK_a \) for this equilibrium is 7.64.\textsuperscript{170} Thus at pH values below ca. 7, 3-methylquinazolinium exists predominantly as its covalent hydrate \( (F-H^+) \) and therefore it is not possible to measure \( pK_{ROH} \) values for the cation \( (D) \) directly.

Addition of partially purified rabbit liver aldehyde oxidase to a solution of 3,4-dihydro-4-hydroxy-3-methylquinazoline at pH 7 (i.e. 85\% of species \( (F-H^+) \) and 15\% of species \( (F) \)) results in a change in the u.v. spectrum (see Figure 5.9). This procedure was repeated in the presence of the potent inhibitor menadione but in this case no such change was observed. These finding suggest the pseudobase to be a substrate for aldehyde oxidase. Incubations with aldehyde oxidase at pH 6.7 (i.e. dealing almost exclusively with species \( (F-H^+) \)) or pH 8.7 (i.e. dealing almost exclusively with species \( (F) \)) produced changes in the u.v. spectrum similar to those observed at pH 7. This tends to suggest that both the pseudobase \( (F) \) and its protonated form \( (F-H^+) \) can act as substrates.
Figure 5.9. The u.v. spectrum of 3,4-dihydro-4-hydroxy-3-methylquinazoline and of the product of its oxidation catalysed by aldehyde oxidase.

(a) --- $10^{-4}$ M solution of 3,4-dihydro-4-hydroxy-3-methylquinazoline in pH 7 phosphate buffer.
(b) --- As (a) plus rabbit liver aldehyde oxidase, incubated for 30 mins at 30°C.
(c) --- As (b) with $2 \times 10^{-5}$ M menadione.

Figure 5.10. The u.v. spectrum of 3-methyl-4-quinazolinone
The u.v. spectrum of the enzymatic oxidation product is the same as that recorded for 3-methyl-4-quinazolinone (G) (Figure 5.10).

The possibility that oxidation had in fact occurred at C-2 to yield the pseudobase (B) was eliminated since the u.v. of this compound was very different from that observed for the incubation product.

Thus from preliminary studies it would appear that aldehyde oxidase is capable of catalysing the dehydrogenation of the pseudobase 3,4-dihydro-4-hydroxy-3-methylquinazoline.
5.6 Discussion

In this chapter aldehyde oxidase has been shown to be capable of catalysing the oxidation of 3,4-dihydro-4-hydroxy-3-methyl-2-quinazolinone and 3,4-dihydro-4-hydroxy-3-methylquinazoline. These are the first reported examples of pseudobases acting as substrates.

It is interesting to note that Brandänge and Lindblom\textsuperscript{50} suggested that the iminium ion (1a), rather than the pseudobase (1b), was the substrate for aldehyde oxidase in the formation of cotinine (2) (see section 1.2.2).

They studied the pH dependence of the inhibitory action of (1) on the enzymatic oxidation of N\textsuperscript{1}-methylnicotinamide. Since $K_m$ and $V_{\text{max}}$ values for N\textsuperscript{1}-methylnicotinamide only underwent a 50\% and two-fold increase respectively, from pH 7.45 to 9.20, it was assumed that a similar small change would be observed for the inhibitory form. The set of $K_i$ values calculated for (1a) was regarded as more plausible than the set for (1b) and hence the conclusion that the charged species (1a) rather than (1b) acted as the substrate. In view of the current finding, that pseudobases can be oxidised by aldehyde oxidase, then the possibility arises that perhaps both (1a) and (1b) are substrates, a situation not considered by these workers.
In 1973, Felsted et al. suggested that aldehyde oxidase catalysed oxidation of \(N^1\)-methylnicotinamide might involve attack by a hydroxyl ion either at C-2 or C-4 forming the respective pseudobases.

![Pseudobases](image)

The ratio of formation of (3):(4) was postulated to be a function of the active site environment. The oxidative step, to the corresponding pyridone, was viewed as being identical for both pseudobases.

Pseudobase formation from heteroaromatic cations is intimately related to covalent hydration of heteroaromatic molecules. There has been some discussion in the literature concerning the possibility of covalent hydration playing a part in the mechanism of hydroxylation catalysed by aldehyde oxidase and xanthine oxidase. For many years, hydration of the substrate was considered to take place followed by dehydrogenation. However in 1966, Fridovich working with xanthine oxidase, showed that with aldehydes the non-hydrated form is that which undergoes catalytic reaction and not the hydrated form which in fact was later shown to be an inhibitor. Subsequently mechanisms have favoured hydroxylation resulting from removal of a hydride ion (or a proton and 2 electrons) from the substrate, leaving a carbonium ion which then reacts with a hydroxyl ion from water (see section 1.8). However in 1980, Davis revived the hydration hypothesis, proposing a general mechanism which involved the inducement of covalent hydration of the substrate by the enzyme (see p.212).
The findings reported in this chapter, i.e. that pseudobases can act as substrates for aldehyde oxidase, support the hypothesis that pseudobases may be intermediates in the enzymatic oxidation of N-heterocyclic cations. In view of this, and from the discussion above, perhaps one could envisage the enzymatic oxidation of quaternary and non-quaternary substrates being represented as shown below:

\[
\begin{align*}
\text{NHz} & \quad \text{H}_{2}\text{O} \quad \rightarrow \quad \text{NHOH} \quad -2\text{H} \\
\text{Covalent hydrate} \\
\text{OH} & \quad \text{R} \\
\text{N} & \quad \text{R} \\
\text{Pseudobase}
\end{align*}
\]

The above scheme may go some way towards explaining why with increase in pH, enzyme activity increases with cationic substrates but not with uncharged substrates or pseudobases.

Normally aldehyde oxidase catalysed oxidation results in hydroxylation of substrate, but oxidation of the pseudobases involves only dehydrogenation.
In the present study, 6-methylpurine was found to competitively inhibit the oxidation of the pseudobase (B) and thus it would appear that both pseudobases and hydroxylatable substrates act as the same site on the enzyme. Further studies with the pseudobases may help to gain a greater insight into the mechanism of action of the molybdenum hydroxylases.

CONCLUDING REMARKS

The work presented in this thesis has brought to light a number of new and interesting findings regarding the interaction of aldehyde oxidase with N-heterocyclic cations. This is the first time that 4-quinolones have been shown to be oxidation products of quinolinium compounds, being formed either simultaneously along with the 2-quinolones or exclusively from cations with a blocked 2-position.

The ratio of 2- to 4-quinolone production from a single substrate was found to be species dependent; the proportion of 4-quinolone was greater with guinea pig enzyme then with rabbit enzyme. Furthermore the bulkier the substituent on the ring nitrogen, the greater the proportion of 4-quinolone formed. Inhibition studies indicated that both 2- and 4-quinolone formation occurs at a common site on the enzyme and it was postulated that different orientations of the substrate molecule at a single active site accounts for the formation of the two isomeric products. However, the possibility that isoenzymes were responsible for production of the isomers could not be eliminated.

The N-heterocyclic cations studied were generally found to be good substrates for rabbit liver aldehyde oxidase, for example N-phenyl-2-phenylquinolinium ($K_m < 10^{-5}$ M) is one of the best substrates known for this enzyme. It might be expected that because of their greater susceptibility to nucleophilic attack, quaternised compounds should be better substrates for aldehyde oxidase than their non-quaternised
analogues, but it is interesting to note that this was not found to be the case for N-methylisoquinolinium or N-methylphenanthridinium.

In general, guinea pig enzyme was found to have a greater affinity for N-heterocyclic cations than rabbit enzyme. Furthermore, a difference was observed in the interaction of the two enzymes with quinolinium compounds possessing a phenyl substituent in position 2 or 4: with rabbit enzyme these compounds are readily oxidised but in contrast they were only extremely slowly oxidised with guinea pig enzyme, although in this case they were found to be very good competitive inhibitors of other substrates. With either enzyme, the ability to bind N-heterocyclic cations appeared to increase with the number of benzene rings in the compound; for example the tetracyclic ethidium bromide is an extremely potent inhibitor for both enzymes \( K_i = 4.6 \times 10^{-6} \) M.

Inhibition studies showed that both quaternary and non-quaternary compounds act at a common site on the enzyme and, from variation of \( \log \left( V_{\text{max}} / K_m \right) \) with pH, an ionisable group on the enzyme, important for binding both types of substrate, was identified with \( pK \approx 8 \). Furthermore it was shown that protonation of this group hindered binding of quaternary cations but enhanced binding of non-quaternary compounds.

Finally, the observation that two pseudobases are substrates for aldehyde oxidase, not only opens up a new class of compound for investigation but also supports the hypothesis that pseudobases are intermediates in the enzymatic oxidation of N-heterocyclic cations.

The findings reported in this thesis should provide additional information on which to base a mechanism of action for aldehyde oxidase, and will be useful in gaining a better understanding of the nature of the substrate binding site.
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