Simultaneous formation of 2- and 4-quinolones from quinolinium cations catalysed by aldehyde oxidase

Susan M. TAYLOR, Christine STUBLEY-BEEDHAM and J. Godfrey P. STELL

Department of Pharmaceutical Chemistry, University of Bradford, Bradford, West Yorkshire BD7 1DP, U.K.

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Quinolinium salts were incubated with partially purified aldehyde oxidase, and the products were separated by high-pressure liquid chromatography and fully characterized by u.v. spectroscopy, i.r. spectroscopy and mass spectrometry. Oxidation of *N*-methylquinolinium salts with either rabbit or guinea-pig liver aldehyde oxidase *in vitro* gave two isomeric products, *N*-methyl-4-quinolone and *N*-methyl-2-quinolone. Incubation of *N*-phenylquinolinium perchlorate similarly yielded two oxidation products, *N*-phenyl-4-quinolone and *N*-phenyl-2-quinolone. The ratio of 2- to 4-quinolone production was species-dependent, the proportion of 4-quinolone with the guinea-pig enzyme being greater than that obtained with the rabbit liver enzyme. Kinetic constants were determined spectrophotometrically for both the quinolinium salts and a number of related quaternary compounds. In general, quaternization facilitated oxidation of a substrate, but a number of exceptions were noted, e.g. *N*-methylisoquinolinium and *N*-methylphenanthridinium. K_m values varied with the nature of electron acceptor employed, and this difference was more marked for quaternary substrates than the unquaternized counterparts. 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 values and in the presence of a competing substrate, *N*-methylphenanthridinium. This may indicate that a quaternary substrate binds to aldehyde oxidase in two alternative positions.

The hepatic enzyme aldehyde oxidase EC 1.2.3.1 is known to oxidize a wide variety of substrates, including aldehydes and diverse azaheterocycles (Coughlan, 1980). Oxidation of aromatic heterocyclic compounds usually occurs at a carbon atom adjacent to a ring nitrogen atom (e.g. C-2 in quinoline) and involves nucleophilic attack by either a hydroxyl ion or a group at the enzyme active site. Quaternization of the ring nitrogen atom, which makes the adjacent carbon atom even more susceptible to nucleophilic attack, also appears to facilitate the reaction. Thus *N*¹-methylnicotinamide is a reasonable substrate, whereas the enzyme shows little activity towards nicotinamide or pyridine (Krenitsky *et al.*, 1972). Similarly, studies with *N*-alkylquinolinium salts have shown that these compounds are oxidized at a

much higher rate than is the parent compound quinoline (Knox, 1946; Hashimoto *et al.*, 1960). Rajagopalan & Handler (1964) found the K_m values for *N*-methylquinolinium and *N*-ethylquinolinium to be of the order of 10^{-4} M compared with 3×10^{-3} M for quinoline. These authors also stated that each quinolinium salt gave rise to a single oxidation product, namely the corresponding 2-quinolone.

*N*¹-Methylnicotinamide has received considerable attention in the past, and likewise was originally thought to give only one oxidation product with aldehyde oxidase, i.e. *N*-methyl-2-pyridone-5-carboxamide (Knox & Grossman, 1948). However, 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 *in vitro* and *in vivo* (Felsted & Chaykin, 1967). Although hydroxylation at positions other than that adjacent

Abbreviation used: h.p.l.c., high-pressure liquid chromatography.

to a nitrogen atom is unusual with this enzyme, it is not unknown, e.g. the quaternary compound 1-(4-pyridyl)pyridinium is oxidized to 1-(4-pyridyl)-4-pyridone (Rajagopalan & Handler, 1964), and cinnoline gives 4-hydroxycinnoline (Stubley *et al.*, 1979). In the case of *N*¹-methylnicotinamide, Felsted & Chaykin (1967) found that the ratio of 2-pyridone to 4-pyridone depends on the species from which the aldehyde oxidase is derived, but is relatively constant for enzyme from any one species under a variety of different conditions. This was interpreted as being indicative of a single enzyme capable of catalysing the oxidation of *N*¹-methylnicotinamide at two alternative positions.

The simultaneous 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 u.v. spectroscopy, which does not distinguish between the 2- and 4-pyridone. Similar considerations may account for the fact that only 2-quinolones have previously been reported as oxidation products of the aldehyde oxidase-catalysed oxidation of *N*-methyl- and *N*-ethyl-quinolinium compounds.

The present paper communicates the findings of a study of the oxidation of *N*-methyl- and *N*-phenyl-quinolinium salts with aldehyde oxidase *in vitro* and presents evidence to show that both 2- and 4-quinolones are produced in the reaction.

Materials and methods

Chemicals

N-Methyl-2-quinolone (Perkin & Robinson, 1913), *N*-methyl-4-quinolone (Späth & Kolbe, 1922), *N*-phenylquinolinium perchlorate (Mortelmans & Van Binst, 1978) and *N*-phenyl-2-quinolone (Manimaran & Ramakrishnan, 1979) were all synthesized by using literature methods.

N-Methylation was carried out by refluxing the appropriate heterocyclic base with methyl iodide in ethanol. The iodide salts were converted into the corresponding chlorides either by passing them down a Dowex 1-X8 column (BDH Chemicals) or by refluxing them with AgCl. The perchlorates were obtained by adding an aqueous solution of the iodide to a solution of NaClO₄. These conversions were effected because iodide ions interfere with ferricyanide as the electron acceptor in enzymic reactions (Bunting *et al.*, 1980). All the chemicals produced were fully characterized by melting

point, together with i.r. spectroscopy, n.m.r. and mass spectrometry.

The synthesis of *N*-phenyl-4-quinolone picrate has been previously reported (Ittyerah & Mann, 1958), but that of its free base has not. *N*-Phenyl-4-quinolone was synthesized from *N*-phenyl-1,2,3,4-tetrahydro-4-oxoquinoline (Cookson & Mann, 1949) by refluxing 0.5 g of the latter with 0.25 g of 5% palladized charcoal in 20 ml of ethanediol for 30 min. The combined filtrate and washings, when evaporated under reduced pressure, gave a red oil. Water was added to the oil, and the *N*-phenyl-4-quinolone was extracted with diethyl ether. The ether was evaporated to yield a white solid. The new compound *N*-phenyl-4-quinolone had m.p. 125°C (from ether) (Found: C, 81.4, H, 5.1, N, 6.3; C₁₅H₁₁NO requires: C, 81.5, H, 5.0, N, 6.3%); mass spectra (70 eV, source temperature 200°C) *m/z* 221 (*M*⁺), 193 (*M*⁺ - CO); i.r. (KBr disc) ν (cm⁻¹) CO, 1625; n.m.r. δ (p.p.m.) ([²H]chloroform) 6.38 (1 H, d), 7 (1 H, m), 7.47 (8 H, m), 8.48 (1 H, m).

Enzyme purification procedure

Partially purified aldehyde oxidase from two sources, New Zealand White female rabbits and Dunkin-Hartley female guinea pigs, was prepared by the procedure described by Stubley *et al.* (1979). Liver homogenates were heat-treated and precipitated by the addition of solid (NH₄)₂SO₄ to give 50% saturation. The precipitate was dissolved in 10–15 ml of 0.1 mM-EDTA and further purified at 0–5°C as follows.

A 10 ml portion of the (NH₄)₂SO₄ fraction was applied on to a Sephadex G-75 (Pharmacia Fine Chemicals) column (50 cm × 1.5 cm internal diam.) and eluted with 0.1 M-EDTA. Active fractions were combined and concentrated by ultrafiltration (Millipore CX10 units) and applied to a DEAE-cellulose (Sigma Chemical Co.) column (50 cm × 1.5 cm internal diam.). The column was eluted with 100 ml of 0.1 M-potassium phosphate buffer, pH 7.8, containing 0.1 mM-EDTA, followed by 0.2 M-potassium phosphate buffer, pH 7.8, also containing 0.1 mM-EDTA. Aldehyde oxidase activity was eluted with the 0.2 M buffer, and the fractions were again concentrated by ultrafiltration. The additional chromatographic procedures represent a 10-fold increase in purity over the (NH₄)₂SO₄ fraction, although a number of other proteins can be seen to be still present when the DEAE-cellulose fraction is subjected to polyacrylamide-gel electrophoresis.

Determination of enzyme activity and Michaelis-Menten constants

All kinetic work and u.v. spectra were recorded by using a Pye-Unicam SP.8-250 spectrophoto-

meter, fitted with a thermostatically controlled cell holder that was kept at 30°C for all rate measurements. Kinetic determinations were carried out in 67mM-potassium phosphate buffer, pH7, containing 0.1mM-EDTA, in a final volume of 3ml. The activity of the enzyme fractions during the purification procedure was tested by monitoring the increase in absorbance at 322nm due to the production of 9-phenanthridone from phenanthridine (50 μ M). Michaelis-Menten constants were determined by measuring the initial rates of oxidation over the first 2min by monitoring either the reduction of horse heart ferricytochrome *c* type III at 550nm (20 μ M) or the reduction of $K_3Fe(CN)_6$ at 420nm (1mM). Specific activities, as shown in Table 2, are expressed as μ mol of *N*-methylquinolinium perchlorate transformed/min per mg of protein. The reaction was monitored with $K_3Fe(CN)_6$ (1mM) as the electron acceptor and a substrate concentration of 10mM. All the initial rates measured were found to be linear over the first 2min of reaction.

Protein concentration was determined by the method of Bradford (1976).

Enzyme incubations

Unless otherwise stated, incubations were carried out with the aldehyde oxidase resulting from the $(NH_4)_2SO_4$ fractionation step. Incubations were also carried out with xanthine oxidase (Sigma Chemical Co.; 0.1 unit/mg of protein).

Incubations for product isolation and identification were carried out in 67mM-potassium phosphate buffer, pH7, containing 0.1mM-EDTA, with a substrate concentration of 8mM and enzyme equivalent to 1g of liver in a total volume of 11.25ml. These mixtures were agitated in a shaking water bath at 37°C (open to the atmosphere) for 1h. The reaction was terminated by the addition of sufficient Na_2SO_4 to precipitate the protein, followed by heating on a boiling-water bath for 20min. Extractions were carried out with dichloromethane (4 $\times$ 25ml), which was concentrated down to a final volume of either 1ml or 5ml. Controls were performed by incubating the substrate or expected metabolites with denatured enzyme (heated at 100°C for 10min).

Determination of product ratios under a variety of conditions was carried out with *N*-methylquinolinium perchlorate as the substrate (0.25mM or 2.5mM unless otherwise stated) and rabbit liver aldehyde oxidase in a total volume of 5ml. The solutions were incubated as described above and the reaction terminated by the addition of 5ml of methanol. This precipitates the protein and also inhibits aldehyde oxidase (Rajagopalan & Handler, 1964). The reaction mixtures were centri-

fuged at 3000g for 5min, and samples of the aqueous methanolic supernatant were injected directly on to an h.p.l.c. column.

Chromatography

Samples (20 μ l) of the dichloromethane extracts from incubations, along with authentic samples, were applied to thin-layer plates coated with Silica Gel G (Merck) containing a fluorescent indicator for irradiation at 254nm. Two solvent systems were employed: solvent A, isopentyl acetate; solvent B, butan-1-ol/acetic acid/water (3:1:1, by vol.).

H.p.l.c. analysis was carried out at ambient temperature by 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 μ l).

Adsorption system. The oxidation products of *N*-methylquinolinium salts were separated on a silica column (25cm $\times$ 4.6mm internal diam.) packed with Shandon Hypersil (5 μ m particle size) as the stationary phase and ethanol/cyclohexane (7:3, v/v) as the mobile phase. The system used for separation of the *N*-phenylquinolones was a silica column (25cm $\times$ 4.6mm internal diam.) with Dupont Zorbax (5 μ m particle size) packing and propan-2-ol/cyclohexane (1:4, v/v) as the eluting solvent. In each case the flow rate was 2ml/min and the eluent was monitored at 325nm. Fractions corresponding to the observed peaks were collected and evaporated to dryness. Residues obtained in this manner were subjected to examination by mass spectrometry and i.r. spectroscopy. Mass-spectrometry determinations were carried out with an AEI model MS 902 instrument, with direct probe insertion. The i.r.-absorption spectra were obtained by using a KBr micro-disc technique and a Perkin-Elmer 297 spectrometer fitted with a KBr beam condenser and attenuator.

Partition system. The incubation products of both *N*-methyl- and *N*-phenylquinolinium salts were also separated on a reverse-phase column (25cm $\times$ 4.6mm internal diam.) and pre-column (5cm $\times$ 4.6mm internal diam.) both packed with Shandon Hypersil ODS (5 μ m particle size). The mobile phase in this case was methanol/6.7mM-potassium phosphate buffer, pH7 (1:1, v/v), with a flow rate of 2ml/min. This system was used for direct injection of incubation solutions and to determine the u.v.-absorption spectra of the oxidation products. The eluate was collected and the u.v.-absorption spectra were recorded in the mobile phase and also after adjustment to pH1 with conc. HCl.

Results and discussion

Identification of metabolites

Preliminary analysis by t.l.c. of the extracts resulting from the incubation of *N*-methyl- and *N*-phenyl-quinolinium salts with aldehyde oxidase indicated the formation of two oxidation products in each case.

H.p.l.c. systems were developed on an adsorption column to separate these products, and the traces obtained (Fig. 1) show quite clearly that two oxidation products are obtained from each substrate. Chromatographic and spectral data relating to these derivatives are given in Table 1. The identities of the four compounds were established as *N*-methyl-2-quinolone, *N*-methyl-4-quinolone, *N*-phenyl-2-quinolone and *N*-phenyl-4-quinolone, by comparison of their properties with those of authentic samples. Therefore oxidation of the *N*-methylquinolinium cation also occurs at position 4

and not only at carbon 2, as previously reported (Rajagopalan & Handler, 1964).

As shown in Fig. 1, the ratio of the 2- to the 4-quinolone depends on both the source of the enzyme and the substrate. It can be seen that (i) the larger phenyl group increases the proportion of the 4-quinolone with both guinea-pig and rabbit aldehyde oxidases and (ii) guinea-pig aldehyde oxidase gives a higher proportion of 4-quinolone than does the rabbit enzyme with both substrates. Felsted *et al.* (1973) noted that replacement of the methyl group in *N*¹-methylnicotinamide by the larger propyl group resulted in a higher proportion of 4-pyridone with both rabbit and pig liver aldehyde oxidases.

Identification of the enzyme system

Controls were carried out to eliminate other possible enzymic or non-enzymic pathways of

Fig. 1. H.p.l.c. traces of 2- and 4-quinolones. Incubations of *N*-methylquinolinium [(a) and (b)] and *N*-phenylquinolinium [(c) and (d)] with rabbit [(a) and (c)] or guinea-pig [(b) and (d)] aldehyde oxidase. 2-Quinolone/4-quinolone ratios: (a) 21:1; (b) 10:1; (c) 4.5:1; (d) 0.075:1.

Table 1. Identification of oxidation products of *N*-methyl- and *N*-phenyl-quinolinium salts with aldehyde oxidase

	U.v. spectra: $\lambda_{\max}$. (nm)		I.r. spectra: ν (CO) (cm^{-1})	Mass spectra: (m/z)	T.l.c.: $100 \times R_F$
	pH 7	pH 1			
<i>N</i> -Methylquinolinium					
Incubation Product 1	326, 270	No change	1650	159, 131	69 (B)
Incubation Product 2	336, 323	307	1620	159, 131	31 (B)
<i>N</i> -Methyl-2-quinolone	326, 270	No change	1650	159, 131	69 (B)
<i>N</i> -Methyl-4-quinolone	336, 326	308	1620	159, 131	31 (B)
<i>N</i> -Phenylquinolinium					
Incubation Product 1	326, 273	No change	1660	221, 193	38.6 (A)
Incubation Product 2	335, 323	320	1625	221, 193	11.3 (A)
<i>N</i> -Phenyl-2-quinolone	326, 273	No change	1660	221, 193	38.6 (A)
<i>N</i> -Phenyl-4-quinolone	335, 323	320	1625	221, 193	11.3 (A)

oxidation that might account for the hitherto unexpected simultaneous production of the 4-quinolones along with the 2-isomers.

Table 2 shows the result of purification of aldehyde oxidase, from crude liver slices to the highly purified DEAE-cellulose stage, on the product ratios with *N*-methylquinolinium perchlorate as substrate. The ratio was found to be constant (within experimental error) throughout the purification procedure. This eliminates the possibility of oxidation by the microsomal mono-oxygenase system, which is destroyed during the initial heating stage at 60°C. Any xanthine oxidase present is separated from the aldehyde oxidase at the DEAE-cellulose step of the 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 Fig. 2.

The u.v.-absorption spectrum obtained when guinea-pig aldehyde oxidase had been incubated for 30 min with *N*-phenylquinolinium 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 the rabbit enzyme, where *N*-phenyl-2-quinolone is the major contributor to the final u.v.-absorption 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 products in various amounts when a substrate is oxidized by aldehyde oxidase under aerobic conditions (Fridovich, 1970). Incubations carried out with the substrate *N*-methylquinolinium perchlorate (a) in the presence of catalase (1 mg/5 ml) or (b) under anaerobic conditions with ferricyanide as the electron acceptor give the same product ratios as in previous experiments. Therefore removing

Table 2. Ratio of 2- to 4-quinolone production on enzyme purification

Stage	Protein (mg)	Specific activity (μmol/min per mg of protein)	Total product yield (%)	2/4-Quinoline ratio ± 1 (n = 6)
Liver slices	200	—	28	17
(NH ₄) ₂ SO ₄ precipitation	29	0.275	78	18
Sephadex G-75	10	0.346	91	21
DEAE-cellulose	0.45	2.84	12.5	17

Fig. 2. U.v. spectra of incubation mixtures of *N*-phenylquinolinium and (a) guinea-pig or (b) rabbit aldehyde oxidase (i) —, 0.1 mM solution of *N*-phenylquinolinium in phosphate buffer, pH 7; (ii) —, as (i) plus aldehyde oxidase incubated for 30 min at 30°C; (iii) ·····, as (ii) with 20 μM-menadione.

peroxide as in (a) or preventing the formation of both superoxide and peroxide (b) did not alter the amount of either product.

Kinetics

All the K_m values reported in Table 3 were determined by monitoring the reduction of an artificial electron acceptor, with either ferricyanide, which reacts at one of the iron-sulphur centres in the enzyme, or cytochrome *c*, which is reduced by the

superoxide anion arising during the aerobic re-oxidation of the enzyme. Owing to the similarity in $\lambda_{\max.}$ of the u.v. absorption by the 2- and 4-quinolones, it is not possible to monitor their formation independently in the same solution by direct spectrophotometric measurement. The rate of reduction of an artificial electron acceptor monitors total product formation, which is equivalent to substrate consumed during reaction and is therefore suitable for K_m determinations.

Table 3. *Michaelis-Menten constants for azaheterocycles with aldehyde oxidase*

K_m values were determined at pH 7 at 30°C with ferricyanide (F), cytochrome *c* (C) or oxygen (O_2) as electron acceptor. Key to species: R, rabbit; G, guinea pig.

Substrate	Species	K_m (M)	Substrate	Species	K_m (M)
Quinoline	R	3.1×10^{-3} (F)	Phenanthridine	R	$< 10^{-6}$ (O_2)†
	R	1.3×10^{-3} (C)
R	1.6×10^{-3} (F)		R	1.8×10^{-4} (F)
	R	2.3×10^{-4} (C)			
	G	5.0×10^{-4} (F)			
	G	2.6×10^{-4} (C)			
<i>N</i> -Methylquinolinium			<i>N</i> -Methylphenanthridinium
R	7.1×10^{-5} (F)	5,6-Benzoquinoline	R	— (F)†
	R	5.6×10^{-6} (C)			
	G	7.8×10^{-5} (F)		R	2.5×10^{-4} (F)
	G	2.2×10^{-5} (C)			
<i>N</i> -Phenylquinolinium			<i>N</i> -Methyl-5,6-benzoquinolinium		
			7,8-Benzoquinoline	R	— (F)†
			R	2.3×10^{-4} (F)
			<i>N</i> -Methyl-5,6-benzoquinolinium		
Isoquinoline	R	2×10^{-4} (F)*	Nicotinamide	R	— (F)
R	— (F)		R	1.3×10^{-3} (F)
	R	— (C)			
<i>N</i> -Methylisoquinolinium			<i>N</i> ¹ -Methylnicotinamide		

* Stubley *et al.* (1979).

† Stubley & Stell (1980).

The progress of the reaction can also be followed by using h.p.l.c. to separate and measure the amounts of each oxidation product formed in a given time. Thus a progress curve may be obtained for the rate of formation of either the 2- or the 4-quinolone. When this was done for *N*-methyl-quinolinium perchlorate, it was seen that while the substrate concentration fell from 1.3 to 0.43 mM over a period of 1 h the ratio of 2- to 4-quinolone remained constant, and therefore the K_m values for the formation of each product are assumed to be equal. Furthermore, all the Lineweaver-Burk plots used for K_m determinations were found to be linear over the substrate concentrations employed ($0.1 \times$ to $10 \times K_m$) with correlation coefficients ≤ 0.997 . A deviation from linearity would have been expected if they were composite plots arising from two K_m values. Felsted & Chaykin (1967) found the K_m values for both products of *N*¹-methylnicotinamide oxidation to be equal for several sources of aldehyde oxidase.

It was found that the values of the Michaelis constants obtained for *N*-methyl- and *N*-phenyl-quinolinium perchlorates depended on the electron acceptor employed. Thus the K_m values obtained for the rabbit liver enzyme with cytochrome *c* were approx. 12-fold lower than those found with ferricyanide. This discrepancy appears to be more marked with quaternized substrates than with uncharged ones, as quinoline shows only small variations in K_m with different electron acceptors.

It is noteworthy that Bunting *et al.* (1980), working with xanthine oxidase and *N*-methyl-quinolinium salts, noted that the K_m values he obtained were higher than those reported in earlier work by Greenlee & Handler (1964), but did not relate to the fact that he used ferricyanide as electron acceptor whereas Greenlee & Handler (1964) employed cytochrome *c*.

In the current study, it was found that the choice of electron acceptor had an even greater effect on V_{max} than on K_m values. For *N*-substituted quinolinium salts, the values obtained with cytochrome *c* were only 2.5–5% of that found with ferricyanide for both sources of enzyme. In this case a similar effect was also noted with uncharged substrates; thus quinoline, which is oxidized more slowly than either of the above quaternized derivatives, gave a V_{max} value with cytochrome *c* that was only 13% 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 re-oxidation of the enzyme have been postulated, namely:

Cytochrome *c* is reduced only by the superoxide anions resulting from the univalent reduction of oxygen (eqn. 1a). 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 concentration and the rate of turnover of the enzyme (Fridovich, 1970). This may explain the discrepancies in K_m and V_{max} values found with the different electron acceptors.

Because of their greater susceptibility to nucleophilic attack, quaternized compounds are generally considered to be better substrates for aldehyde oxidase than their unquaternized counterparts. The results in the present study for *N*-methyl- and *N*-phenyl-quinolinium salts and the benzoquinolines support this concept (see Table 3). However, two important exceptions to this generalization were noted. Phenanthridine is an excellent substrate for aldehyde oxidase, but its *N*-methylated analogue is somewhat less efficient in this respect. Furthermore, whereas isoquinoline is a better substrate than the isomeric quinoline, *N*-methylisoquinolinium perchlorate is almost devoid of substrate activity. Thus the relationship between the oxidation of heterocyclic bases and their quaternized cations by aldehyde oxidase is not as simple as originally envisaged.

Investigation of product ratios under various conditions

We have established that aldehyde oxidase is responsible for the formation of both products from quinolinium salts. This raises the question as to whether there is more than one type of active site on the same molecule or even the possibility that isoenzymes are present. 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 (Porter *et al.*, 1977).

Several conditions have already been discussed that had no effect on product ratios. These included purification of the enzyme, variation of substrate concentration, the use of ferricyanide as the electron acceptor, anaerobic incubations and addition of catalase to the system. In addition, storage in liquid N_2 does not alter the product ratio.

Enzyme preparations from rabbits treated with hydralazine show a marked decrease in aldehyde oxidase activity (Johnson *et al.*, 1982). Furthermore, the same authors found that phthalazine (50 mg/kg for 7 days) appeared to cause induction of the enzyme. 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-quinolone/4-quinolone ratio was also investigated. With increasing pH, K_m values for substrates bearing a quaternary nitrogen atom decrease, whereas the converse is true for uncharged substrates (Rajagopalan & Handler, 1964). This was confirmed in the present study: for example, the K_m value for *N*-methylquinolinium perchlorate decreased from 1.56×10^{-3} M at pH 7 to 5.4×10^{-4} M at pH 9.9. With the DEAE-cellulose fraction of the enzyme, it was noted that there was a tendency to produce a greater proportion of the 4-quinolone at higher pH values.

One factor that did bring about a significant change in the 2-quinolone/4-quinolone ratio was the use of a competing substrate. The oxidation of *N*-methylquinolinium perchlorate (2.5 mM) by aldehyde oxidase was monitored in the presence of *N*-methyl phenanthridinium perchlorate (1 mM), when the ratio of 2- to 4-quinolone increased from 17.5 to 29, indicating that 4-quinolone production is inhibited more effectively than that of the 2-quinolone. When the experiment was repeated with much lower concentrations of *N*-methylphenanthridinium an increase in product ratio was still obtained although the difference was less marked. The latter compound was chosen as competing substrate because oxidation can only occur at one position in the heterocyclic ring, i.e. the carbon atom adjacent to the nitrogen atom. However, this observation appears to indicate the presence of two enzymic sites with differing affinities for the substrate *N*-methylphenanthridinium.

In contrast with previous reports, we have isolated and identified two oxidation products from incubations of quinolinium salts with soluble enzyme fractions. The isomeric quinolones are produced simultaneously via the action of aldehyde oxidase, and for each substrate the product ratio remains constant under a variety of conditions, indicating the involvement of a single binding site. However, this ratio does change in the presence of a competing substrate, and

therefore the possibility of two different (alternative) binding sites or two isoenzymes cannot be discounted.

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