On the Oxidation of *N*-Methyl and *N*-Benzylpyrimidin-2- and -4-ones by Rabbit Liver Aldehyde Oxidase [1]

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The presence of a methyl or benzyl group at N-1 or N-3 of 2- and 4-pyrimidone does not affect the site of oxidation by rabbit liver aldehyde oxidase. From all substrates studies only one product *viz.* the corresponding N-1 or N-3 substituted uracil has been obtained. The maximum rates of oxidation by free enzyme show an optimum in the *p*H range 6.5-7.8, which is little influenced by the site and the size of the N-substituent. Application of immobilized enzyme in small scale synthesis gives 1- or 3-R-uracils (R = methyl, benzyl) in 43-78% yield.

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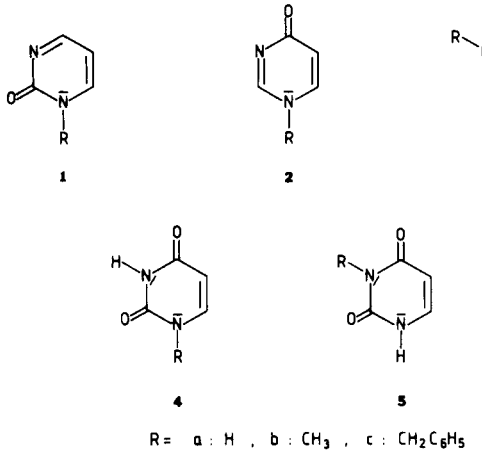
The application of immobilized enzymes in organic synthesis is a subject of current interest in our laboratories. The molybdenum iron-sulfur containing flavoproteins xanthine oxidase/dehydrogenase [2] isolated from bovine milk, chicken liver or Arthrobacter cells and aldehyde oxidase [2] obtained from rabbit liver were employed as immobilized biocatalysts in the oxidation of several six-membered azaheterocyclic compounds [1,3-5]. These enzymes are of particular interest since they possess broad substrate specificities [2]. The oxidation of heteroaromatic cations such as 1-alkyl(aryl)-3-aminocarbonylpyridinium chlorides [4,5] and 1-alkyl(aryl)quinolinium chlorides [1] was investigated using aldehyde oxidase in particular. A marked influence of the size and the electronic effect of the substituent at the ring nitrogen atom on the site(s) of oxidation in these compounds was established.

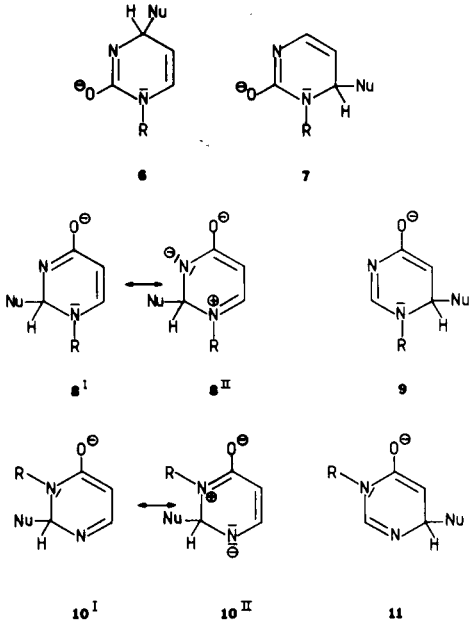
Up to now the reactivity of aldehyde oxidase towards uncharged substrates has hardly been investigated with regard to the influence of *N*-substituents on the site of oxidation [6]. We therefore extended our research to the aldehyde oxidase-mediated oxidation of *N*-methyl- and *N*-benzylpyrimidin-2- and -4-ones (Scheme 1).



Scheme 1

Results and Discussion.

It is known that the compounds 2-pyrimidone and 4-pyrimidone are readily oxidized by aldehyde oxidase [6]. Analysis with hplc showed that substrates **1a-c** and **2a-c** only give formation of the corresponding uracil derivatives **4a-c**. Substrates **3b-c** yield the N-3 substituted compounds **5b-c** as sole products. In all these oxidation reactions no indication is found for the simultaneous formation of products isomeric with **4** or **5** and no barbituric acid or its derivatives are detected either. This is confirmed by incubation of compounds **4** and **5** with aldehyde oxidase; no conversion into barbituric acid or its derivatives is observed. The results indicate that in substrates **1b** and **1c** the position adjacent to the unsubstituted nitrogen atom (C-4) is preferred to the position adjacent to the sub-



Scheme 2

stituted nitrogen atom (C-6) for oxidation. To understand these oxidation patterns we may consider the intermediates formed on addition of the active site nucleophilic species [5] to these substrates (Scheme 2). Nucleophilic attack on substrates **1a-c** can take place either at C-4, yielding intermediates **6a-c** or at C-6, forming adducts **7a-c**. Intermediate **6** possess a *p*-quinoid structure and intermediate **7** an *o*-quinoid structure.

Since in general the *p*-quinoid structure is more stabilized than the corresponding *o*-quinoid structure [7], it is comprehensible that **1a-c** are oxidized at the C-4 position. For 4-pyrimidone it is well documented that the *o*-quinoid structure is more stable than the *p*-quinoid structure [8]. It is expected that from the two possible adducts **10** and **11** obtained from **3**, adduct **10** is the most preferred since in adduct **10** there is more extended conjugation between both nitrogen atoms than in **11**, thus favouring **10** to **11**. The addition of the nucleophile to **2** predicts preferred formation of adduct **8** to **9**, since **8** is more resonance-stabilized than **9**. These results are also in agreement with the observations that the presence of a nucleophilic substituent at C-4 deactivates a nucleophilic attack at C-6 in favor of C-2: for instance 2,6-dichloro-4-methoxypyrimidine only undergoes dechlorination by methoxide ions at C-2 [7].

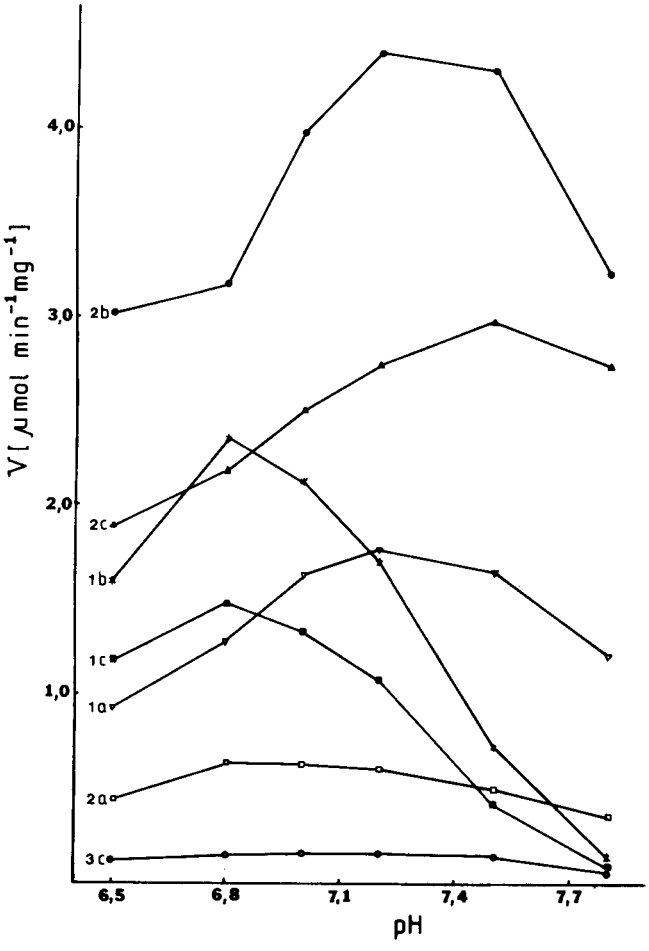


Figure 1. Maximum rates for the oxidation of substrates **1a-c, 2a-c** and **3c** by free aldehyde oxidase as a function of *p*H at 25°.

The maximum oxidation rates for substrates **1a-c, 2a-c** and **3c** with free enzyme are determined as a function of *p*H in the *p*H-region 6.5-7.8 (Figure 1). For compound **3b** we found a rather low oxidation rate at *p*H 7.0 and therefore we did not determine the maximum rate for this substrate over the whole *p*H range. All substrates studied show a *p*H-optimum in the *p*H-region of 6.5-7.8. The *p*H-optima for substrates **1a-c** shift from about *p*H 7.2 for compound **1a** to *p*H 6.8 for **1b** and **1c**. For the 4-pyrimidones substituted at N-1 the optimum shifts to slightly higher *p*H values, whereas substitution at N-3 does not affect the *p*H-optimum significantly. From these observations we conclude that the effect of *N*-substitution by a methyl or a benzyl group on the *p*H-optimum of the maximum rate is rather small. For the pyrimidones **1a** and **2a** the maximum rate of oxidation is found to decrease at higher *p*H, due to deprotonation (**1a**: pK_a 9.37 [9] and **2a**: pK_a 8.60 [9]) leading to negatively charged species which are deactivated for nucleophilic attack [5].

It is interesting that on comparison of the results of these *p*H dependencies of the oxidation rates with those obtained for the cationic substrates studied before [1,5], a remarkable difference in the shape of the *p*H curves is observed. 3-Aminocarbonyl-1-phenylpyridinium chloride [5] and 1-methylquinolinium chloride [1] for instance, exhibit a nearly constant maximum rate above *p*H 7.5, whereas we here find bell-shaped *p*H curves for all substrates.

The magnitude of the maximum rates is strongly dependent on the nature and the position of the *N*-substituent. Upon methylation or benzylation at N-1 of 4-pyrimidone the maximum rate increases drastically. This effect is comparable to that obtained upon introduction of a methyl

Table 1

Kinetic Data for the Oxidation of Substrates **1a-c, 2a-c** and **3b-c** by Free Aldehyde Oxidase at *p*H 7.0 [a]

Substrate	K_M [b,d]	V [c,d]
1a	244	1.63
1b	272	2.13
1c	78	1.33
2a (= **3a**)	543	0.62
2b	185	3.98
2c	66	2.51
3b	156	0.052
3c	61	0.158

[a] The maximum oxidation rate for the reference substrate 3-aminocarbonyl-1-methylpyridinium chloride [4] with this aldehyde oxidase preparation was 0.36 ± 0.02 μmole/minute mg. [b] In μmole/ℓ. [c] In μmole/minute mg. [d] Accuracy for **2a, 3b-c** was 4%, for **1a-b, 2b-c** 7% and for **1c** 14%.

Table 2

Product Yields, Melting Points and Exact Mass Data of Uracil Derivatives **4b-c** and **5b-c** Obtained by Oxidation Using Immobilized Aldehyde Oxidase

Substrate	Product	Yield (%)	Mp (°C)	Formula	Exact Mass Exp	Exact Mass Theor
1b	**4b**	43	231-233 [a]	$C_5H_6N_2O_2$	126.0429	126.0429
1c	**4c**	44	174-176 [b]	$C_{11}H_{10}N_2O_2$	202.0747	202.0742
2b	**4b**	49	230-232 [a]	$C_5H_6N_2O_2$	126.0428	126.0429
2c	**4c**	52	174-176 [b]	$C_{11}H_{10}N_2O_2$	202.0742	202.0742
3b	**5b**	64	178-180 [c]	$C_5H_6N_2O_2$	126.0429	126.0429
3c	**5c**	78	180-182 [d]	$C_{11}H_{10}N_2O_2$	202.0742	202.0742

[a] Lit [9] 232-233°. [b] Lit [13] 173-174°. [c] Lit [9] 179°. [d] Authentic sample: 182-183°.

group in hypoxanthine at the equivalent N-3 position [6]. Introduction of substituents at N-3 of 4-pyrimidone results, irrespective of the size of the substituent, in a large decrease of the maximum rates (Table 1) over the whole *p*H range. These results can be partly explained by comparing the initial and intermediate structures of the substrate in the oxidation reactions. Compounds **2b,c** both possess the *p*-quinoid structure, but intermediates **8b,c** with their azadiene *o*-quinoid structure are strongly resonance-stabilized. This will facilitate the initial nucleophilic attack at C-2 in the oxidation of these substrates. The corresponding step in the oxidation of **2a**, which is present in the *o*-quinoid form **3a** [8] involves intermediate **10a** and the adduct formation certainly requires a higher transition state energy than **2b,c**. For **3b,c** the initial structures are *o*-quinoid and therefore no rate enhancement could be expected in comparison with the oxidation rate for **3a**. On the contrary a substantial decrease in maximum rate has been observed for both compounds. It is apparent that other effects must play an important role and a likely possibility is that substrates **3b,c** are oriented differently in the active site of the enzyme because of interaction of the hydrophobic group at N-3 with the proposed hydrophobic region in the enzyme [4], possibly making C-2 less accessible for nucleophilic attack. Support for this suggestion comes from the considerably lower Michaelis constant values (K_M) observed upon the introduction of large hydrophobic substituents (Table 1).

For 2-pyrimidone and its derivatives the situation is less clear. At low *p*H the reasoning used for 1-R-4-pyrimidones also shows some validity here, but above *p*H 7.2 (R = methyl) and *p*H 6.9 (R = benzyl) the *N*-substituted 2-pyrimidones are oxidized at lower rates than 2-pyrimidone itself. Clearly other effects in the active centre of the enzyme also play a predominant role in the oxidation of these compounds at higher *p*H.

Oxidation of substrates **1b-c, 2b-c** and **3b-c** on a small preparative scale is performed with immobilized aldehyde oxidase. The enzyme preparation used for this purpose contained a low indigenous xanthine oxidase activity as well. Therefore we isolated xanthine oxidase from rabbit liver and tested the activity of this enzyme on these substrates. We established by hplc analysis only very small activities of xanthine oxidase for substrates **1b** and **1c**, which are oxidized into the uracil derivatives **4b** and **4c**, respectively. The other substrates (**2b-c, 3b-c**) are not converted by this enzyme. Consequently the yields in Table 2 for products **4b-c** derived from substrates **1b-c** may contain a very small contribution from the xanthine oxidase-mediated reaction.

The product yields found are comparable with those obtained in the oxidation of 1-alkyl(aryl)quinolinium chlorides [1], although the slowly converted substrates **3b-c** give a signficantly higher product yield. To obtain the products **4b-c** by this method the oxidation of substrates **2b-c** is slightly more profitable than starting from substrates **1b-c**.

EXPERIMENTAL

Mass spectra were determined on an AEI MS 902 mass spectrometer equipped with a VG ZAB console. The hplc analysis was performed with a Varian 5000 instrument equipped with a Micro Pak MCH-10 column (30 × 0.4 cm), a Schoeffel GM-770 monochromator and a SF-770 spectroflow monitor. Operating conditions were Δp 14.8 MPa (2146 psi), eluent water-methanol 80:20 or 70:30 (v/v) and flow rate 1.6 ml/minute. The uv spectra and kinetic assays were determined on an Aminco DW-2a UV/VIS spectrophotometer. Column chromatography was carried out over Merck Silica gel 60 (70-230 ASTM). Partially purified aldehyde oxidase (E.C. 1.2.3.1) was prepared from frozen rabbit livers as described previously [4]. This type of preparation was used for kinetic assays. For synthetic purposes a less-purified preparation, acquired by ommission of the hydroxylapatite step in the purification procedure, was employed. Rabbit liver xanthine oxidase (E.C. 1.2.3.2) was isolated as described before [1].

Starting Materials and Reference Compounds.

The following compounds were synthesized according to procedures described in the literature: 4-pyrimidone (**2a**) [10], 1-methyl-4-pyrimidone (**2b**) [10], 1-benzyl-4-pyrimidone (**2c**) [10], 3-methyl-4-pyrimidone (**3b**) [10], 3-benzyl-4-pyrimidone (**3c**) [10], 1-methyl-2-pyrimidone (**1b**) [11], 1-benzyl-2-pyrimidone (**1c**) [12], 1-methyluracil (**4b**) [9], 3-methyluracil (**5b**) [9], 1-benzyluracil (**4c**) [13], 3-benzyluracil (**5c**) [14], 3-aminocarbonyl-1-methylpyridinium chloride [4].

2-Pyrimidone, 4,6-dihydroxypyrimidine, uracil and barbituric acid were purchased from Aldrich and purified by recrystallization from appropriate solvents.

HPLC Analysis.

The hplc analysis of incubation mixtures was performed as described previously [4]. The substrate concentration in the mixture was 0.2 m*M* and 25 m*M* potassium phosphate, *p*H 7.5 containing 0.1 m*M* EDTA was employed as a buffer. Registration of the oxidation products was carried out by uv detection at 254 nm (log ϵ for **4a**: 3.90; **4b**: 3.80; **4c**: 3.89; **5b**: 3.83; **5c**: 3.83; 4,6-dihydroxypyrimidine: 3.89; barbituric acid: 4.24).

Synthesis of the Uracil Derivatives **4b-c** and **5b-c** by Immobilized Aldehyde Oxidase.

Rabbit liver aldehyde oxidase was immobilized by absorption onto DEAE Sepharose CL 6B as described before [4], applying 45 mg of aldehyde oxidase-containing protein per 3.5 ml packed Sepharose gel during immobilization. The immobilized enzyme preparation was packed in a column and washed with 10 m*M* potassium phosphate buffer, *p*H 7.5 (+ 0.1 m*M* EDTA) at 4°. For each conversion 18 units of aldehyde oxidase were used (for definition, see Kinetic Assays). The DEAE Sepharose was regenerated as described elsewhere [15]. A solution of 28 mg substrate in 500 ml 10m*M* phosphate buffer, *p*H 7.5 (+ 0.1 m*M* EDTA) was pumped (45 ml/hour) through the column at 4° and recycled once or twice until the aldehyde oxidase activity was depleted. The formation of product was registrated at a suitable wavelength (see Kinetic Assays). After evaporation of the collected effluent which was acidified to about *p*H 6.5, to dryness, the residue was purified by column chromatography (eluent chloroform-ethanol 9:1 (v/v)). The uv and mass spectra of the isolated products were identical with those of authentic materials. Yields, melting points and exact mass measurement data are collected in Table 2.

Kinetic Assays.

The assay for aldehyde oxidase was carried out as described previously [4] and each assay was performed at least in duplicate. In the *p*H range studied (6.5-7.8) potassium phosphate buffers with an ionic strength, I = 0.05, containing 0.1 m*M* EDTA, were employed [16]. The wavelengths (λ in nm) and corresponding molar differential absorption coefficients (log $\Delta\epsilon$) at *p*H 7.0 are: **1a-4a**: 300 (3.71); **1b-4b**: 302 (3.75); **1c-4c**: 302 (3.76); **2a-4a**: 258 (3.60); **2b-4b**; 273 (3.72); **2c-4c**: 246 (4.15); **3b-5b**; 254 (3.68); **3c-5c** 258 (3.54). These $\Delta\epsilon$ values were corrected for *p*H effects in the region *p*H > 7. For aldehyde oxidase one unit of enzyme activity is defined as the amount of enzyme which oxidized 1 μmole of 3-aminocarbonyl-1-methylpyridinium chloride per minute at 25°. The assay conditions were: 5 m*M* substrate in 50 m*M* potassium phosphate buffer, *p*H 7.8 (+ 0.1 m*M* EDTA) with the reaction being monitored at 292 nm (log $\Delta\epsilon$ 3.64). Kinetic data were calculated from Lineweaver-Burk plots [17].

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REFERENCES AND NOTES

[1] Part **10** in The use of Immobilized Enzymes in Organic Synthesis. For part **9** see S. A. G. F. Angelino, B. H. van Valkengoed, D. J. Buurman, H. C. van der Plas and F. Müller, *J. Heterocyclic Chem.*, in press.

[2] M. P. Coughlan, "Molybdenum and Molybdenum-containing Enzymes", M. P. Coughlan, ed, Pergamon Press, Oxford, 1980, Chapter 4.

[3] J. Tramper, W. E. Hennink and H. C. van der Plas, *J. Appl. Biochem.*, **4**, 263 (1982) and references cited therein.

[4] S. A. G. F. Angelino, D. J. Buurman, H. C. van der Plas and F. Müller, *Rec. Trav. Chim.*, **101**, 342 (1982).

[5] S. A. G. F. Angelino, D. J. Buurman, H. C. van der Plas and F. Müller, *ibid.*, **102**, 331 (1983).

[6] T. A. Krenitsky, S. M. Neil, G. B. Elion and G. H. Hitchings, *Arch. Biochem. Biophys.*, **150**, 585 (1972).

[7] R. G. Shepherd and J. L. Frederick, "Advances in Heterocyclic Chemistry", A. R. Katritzky, A. J. Boulton and J. M. Lagowski, eds, Academic Press, New York, 1965, Vol 4, Chapter 6.

[8a] A. R. Katritzky and J. M. Lagowski, *ibid.*, 1963, Vol 1, p 369. [b] J. Elguero, C. Marzin, A. R. Katritzky and P. Linda, "The Tautomerism of Heterocycles", - "Advances in Heterocyclic Chemistry", Supplement 1, A. R. Katritzky and A. J. Boulton, eds, Academic Press, New York, 1976, Chapter 2.

[9] D. J. Brown, E. Hoerger and S. F. Mason, *J. Chem. Soc.*, 211 (1955).

[10] L. Bauer, G. E. Wright, B. A. Mikrut and C. L. Bell, *J. Heterocyclic Chem.*, **2**, 447 (1965).

[11] J. J. Fox and D. van Praag, *J. Am. Chem. Soc.*, **82**, 486 (1960).

[12] D. J. Brown and J. S. Harper, *J. Chem. Soc.*, 5542 (1965).

[13] B. R. Baker and M. Kawazu, *J. Med. Chem.*, **10**, 302 (1967).

[14] C. W. Whitehad, *J. Am. Chem. Soc.*, **74**, 4267 (1952).

[15] Pharmacia, "Ion Exchange Chromatography, Principles and Methods", Rahms, Lund, 1980-1981.

[16] H. A. McKenzie, "Data for Biochemical Research", A. M. C. Dawson, D. C. Elliott, W. H. Elliott and K. M. Jones, eds, 2nd Ed, Oxford at the Clarendon Press, London, 1969, pp 503.

[17] I. H. Segel, "Enzyme Kinetics, Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems", Wiley-Interscience, New York, 1975, p 46.