THE ENZYMIC OXIDATION OF 2-CHLOROPURINE

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SUMMARY

2-Chloropurine is the first purine, for which simultaneous enzymic oxidation along two different pathways has been established. Attack at either carbon atom 6 or 8 proceeds at the same rate. This phenomenon is ascribed to the mesomeric (+M) effect of the halogen in position 2, which exhibits pronounced aromatic properties.

INTRODUCTION

Recently a hypothesis was advanced to explain the fact that the monohydroxy purines use only one of the two possible alternative pathways of enzymic oxidation leading to uric acid[1]. It was postulated that in the ES complex each derivative assumes a specific "activated" form in which a free NH-group at either position 3 or 9 participates in binding; the "active" form of the molecule then exposes one specific carbon atom to nucleophilic attack by a hydroxyl ion. However, application of this reaction scheme to purine itself met with difficulties because it was found that 7- or 9-methylpurine are also oxidized to the corresponding hypoxanthines. The presence of a free NH-group in the imidazol ring is therefore not essential for activation of carbon atom 6 in purine.

In contrast to purine, pteridine is attacked by mammalian xanthine oxidase (XO) simultaneously at carbon 2 and 4, the latter corresponding to position 6 in the purine ring[2]. The lack of enzymic attack at position 2 in purine may be ascribed to a more rigid localisation of the double bonds in the pyrimidine moiety, due to fixation of the C(4)–C(5) double bond, common to both heterocyclic rings. The requirement of 6 π-electrons for resonance stabilisation of the imidazol ring is being held responsible for this MILLS-NIXON-like effect[3]. In pteridine, fixation of the central double bond may be assumed to be less stringent, because of the mutually opposed polarity of the $\overset{6}{C}=\overset{5}{N}$ and $\overset{7}{C}=\overset{8}{N}$ groups, which detracts from the aromatic character of the pyrazine ring.

On this basis, it may be expected that introduction of polar substituents, which do not alter the basic unsaturated structure of purine, but induce a different distribution of the π-electrons, will make positions other than C-6 susceptible to enzymic attack. It has been observed previously that in the pyrimidine ring a strong electron-donating substituent, such as the 6-dimethylamino group, makes all available carbon

Abbreviations: XO, xanthine oxidase; O.D., optical density.

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atoms refractory[4,5]. On the other hand, 6-chloropurine is oxidised enzymically to 6-chloro-2,8-dihydroxypurine[6], although the pathway of this reaction has not been elucidated. As a suitable test case, we have selected 2-chloropurine (I) because the substituent in position 2 is relatively stable[7–9] and can be expected to survive during the enzymic oxidation. The results obtained in the reaction of I with XO may shed light on the mechanism of the oxidations, catalysed by this enzyme.

MATERIALS AND METHODS

Purine derivatives

2-Chloropurine[9] (I) and its 6-hydroxy derivative[10] (II) were a gift of Dr. J. A. MONTGOMERY, Kettering-Meyer Laboratory, Southern Research Institute, Birmingham, Alabama, USA.

2-Chloro-8-hydroxypurine (III) was synthesized as follows: An intimate mixture of 2-chloro-4,5-diaminopyrimidine[11] (0.5 g) and urea (0.7 g) was heated for 25 min to 200–210°. The cake was extracted repeatedly with hot water. Upon cooling, clusters of colorless plates were deposited. Yield: 0.2 g (30 %); m.p. > 300°.

Anal. calcd. for $C_5H_3N_4OCl \cdot H_2O$: C, 31.9; H, 2.7 %. Found: C, 32.3; H, 2.5 %.

2-Chloro-6,8-dihydroxypurine (IV) is also a new compound. It was obtained as end-product of all enzymic reactions, studied in this paper. The u.v. spectrum of IV is very similar to that of 6,8-dihydroxypurine and is clearly distinguished from uric acid. IV and uric acid also show different R_F values (Table I). Therefore, there can be no doubt that the end-product of oxidation of I, II and III has retained the chlorine atom in position 2.

Enzymes

Highly purified XO from cow's milk was obtained through the courtesy of Professor F. BERGEL and Dr. R. C. BRAY of the Chester Beatty Institute of Cancer Research, London, England. This preparation, when diluted 1:4800, produced at 28° and pH 8.0 1 μg/ml/min of uric acid, while $6.5 \cdot 10^{-5}$ M xanthine served as substrate.

In all experiments, 10 units/ml of catalase (Worthington) was added. This enzyme concentration is capable of decomposing 10 mg/ml/min of H_2O_2, when the latter is approximately $1.5 \cdot 10^{-3}$ M.

Kinetic measurements

The progress of enzymic oxidation was followed at the appropriate wave lengths by the use of a Beckman u.v. spectrophotometer. The temperature was maintained at 28° by a dual thermospacer; 0.01 M phosphate of pH 8.0 served as buffer. Two types of controls were used simultaneously: One quartz cell, containing substrate and buffer only, and a second one, to which also catalase was added. Since none of the purines studied presently underwent any reaction in the absence of XO, the changes of O.D., taking place under the influence of this enzyme, were plotted directly as function of time and the initial rates calculated from the linear portions of the kinetic curves (see Figs. 2 and 4).

Chromatographic analysis

Sheets of Whatman paper No. 1 were first washed according to the procedure of

BERGMANN, DIKSTEIN AND CHAIMOVITZ[12] and then air-dried. The following solvents were used for development by the descending method:

Solvent A, 95 % ethanol–glacial acetic acid–water (85:5:10, v/v/v);

Solvent B, 95 % ethanol–pyridine–water (70:20:10, v/v/v);

Solvent C, isopropanol–dimethylformamide–25 % ammonia (65:25:10, v/v/v).

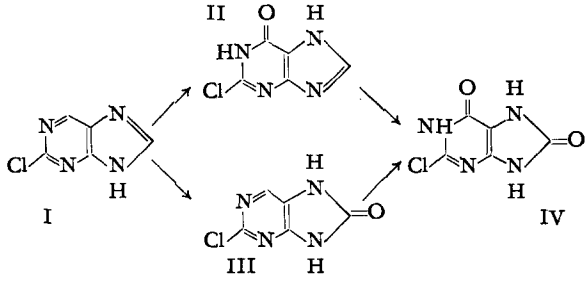
Spots were located by observing fluorescence under a Mineralight u.v. lamp, emitting light of about 255 mμ. They were then cut out and extracted with suitable buffers for determination of absorption spectra.

The pertinent data on the purines, used in this investigation, are collected in Table I. It is evident that replacement of the hydrogen in position 2 by chlorine has a bathochromic effect, which decreases with increasing number of hydroxyl groups in the molecule.

RESULTS

The enzymic oxidation of 6- and 8-hydroxy-2-chloropurine

The possible pathways of the oxidation of 2-chloropurine are indicated in the following scheme:



In order to evaluate the chances to isolate possible intermediates in the conversion of I to IV, it is necessary first to measure the susceptibility of the monohydroxy derivatives II and III to enzymic attack. The absorption spectra in Fig. 1 indicate that oxidation of 2-chloro-6-hydroxypurine to IV can be conveniently followed at 275 and 280 mμ. The changes of O.D., observed at these wave lengths, are plotted in Fig. 2 as function of time. The slope of the initial portion of each curve gave the same rate value. The oxidation of the 8-hydroxy derivative III, however, was too slow to be determined accurately. Therefore, only an approximate value, derived from measurements at 260 and 290 mμ, is given in Table I. The oxidation of the 6-hydroxy derivative is about 40 times more rapid than that of its 8-isomer. This result should be compared to the ratio of formation of uric acid from xanthine and 2,8-dihydroxypurine, respectively, which was determined previously as 500:1 (see ref. 13). The end-product of oxidation of II and III was found identical in every respect.

Oxidation of 2-chloropurine (I)

In Fig. 3, the absorption spectra of 2-chloropurine and its potential oxidation products are recorded. From these curves, 3 wave lengths were selected for rate determinations: (a) At 308 mμ, the isosbestic point of I and II, an initial increase in O.D. will measure the rate of formation of III; (b) at 255 mμ, the isosbestic point of

I and III, formation of II can be recognised by the initial rise in O.D.; (c) finally, at 235 mμ, the two potential intermediates in the oxidation of I possess an isosbestic point, and here any initial increase of O.D. is proportional to the total rate of disappearance of 2-chloropurine, regardless of the pathway used by XO. Fig. 4 represents the results of such measurements. The initial rate values, derived from Fig. 4 and shown in Table II, prove that both intermediates are formed at practically identical speed

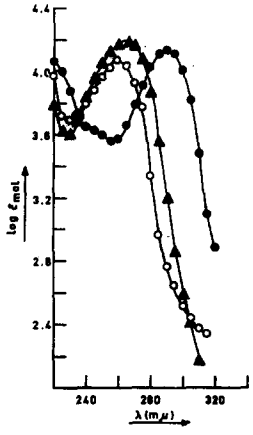


Fig. 1. Ultraviolet absorption spectra, measured in aqueous solution at pH 8.0. ○—○, 2-chloro-6-hydroxypurine (II); ●—●, 2-chloro-8-hydroxypurine (III); ▲—▲, 2-chloro-6,8-dihydroxypurine (IV).

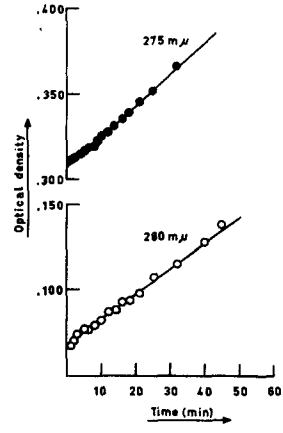


Fig. 2. Changes in O.D. as function of time during the oxidation of 2-chloro-6-hydroxypurine (II). XO, 1:800; substrate, $6 \cdot 10^{-5}$ M; pH 8.0; temperature 28°.

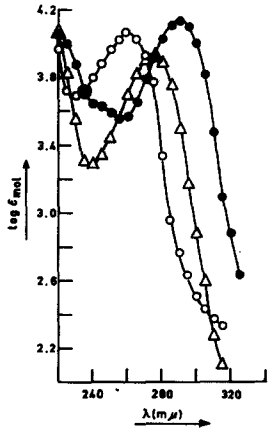


Fig. 3. U.V. absorption spectra of 2-chloropurine (I) and its monohydroxy derivatives at pH 8.0. △—△, 2-chloropurine (I); ○—○, 2-chloro-6-hydroxypurine (II); ●—●, 2-chloro-8-hydroxypurine (III).

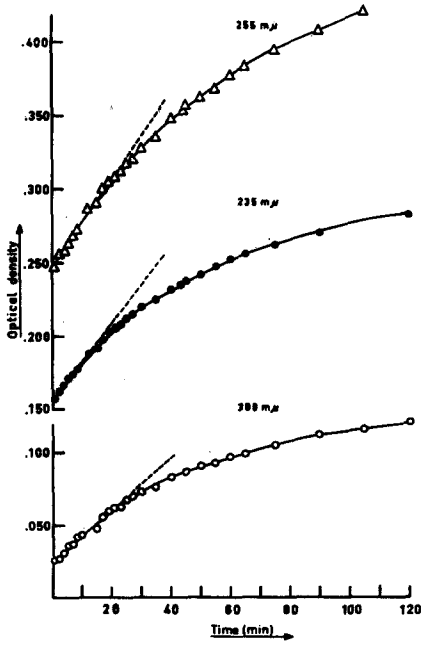


Fig. 4. Changes in O.D. during the enzymic oxidation of 2-chloropurine. XO, 1:1000; substrate, $6.5 \cdot 10^{-5} M$; pH 8.0; temperature 28°. 235 mμ-isosbestic point of II and III (rate of consumption of 2-chloropurine). 255 mμ-isosbestic point of I and III (rate of formation of II). 308 mμ-isosbestic point of I and II (rate of formation of III).

TABLE I

PROPERTIES OF 2-CHLOROPURINE AND ITS HYDROXY DERIVATIVES

Values in parentheses refer to the non-chlorinated analogs and are recorded for comparison.

Compound	*λmax (mμ) at pH 8.0*	*R_F values in solvent*			*Fluorescence*	*Enzyme concentration used*	*Relative rate of oxidation* (xanthine = 100)*
		A	*B*	*C*			
Chloro-purine (I)	275 (263)	0.83 (0.62)	0.78	0.73	Blue-violet	1:1000	3.1 (20)
Chloro-6-hydroxy-purine (II)	259 (252)	0.63 (0.52)	0.37	0.44	Violet	1:800	0.8 (70)
Chloro-8-hydroxy-purine (III)	290 (280)	0.78 (0.61)	0.80	0.66	Sky-blue	1:100	< 0.02 (1.5)
Chloro-6,8-dihydroxy-purine (IV)	265–266 (261)	0.37 (0.34)	—	0.42	Violet		— (100)
ric acid	292	0.26	0.31	—	Dark-violet		—

* Calculated for an enzyme dilution of 1:4800.

TABLE II

EVALUATION OF INDIVIDUAL RATES IN THE ENZYMIC OXIDATION OF 2-CHLOROPURINE

Xanthine oxidase, 1:1000; 2-chloropurine, $6.5 \cdot 10^{-5}$ *M*; pH 8.0; temperature 28°.

	Wavelength observed		
	255 mμ (Formation of II)	*308 mμ (Formation of III)*	*235 mμ (Disappearance of I)*
(a) Observed initial change in O.D. (calculated per hour; from Fig. 4)	0.180	0.106	0.165
(b) Calculated change in O.D. per mole of substrate, $\times 10^{-4}$ (from Fig. 3)	0.70	0.40	0.315
(c) Initial rate = (a)/(b) (μmoles/h)	25.7	26.5	52.4
(d) Relative rate (%) (xanthine = 100)	1.49	1.53	3.08

and that indeed the rate of consumption of substrate I (column 4) is very nearly equal to the sum of the rates of formation of II (column 2) and III (column 3).

Chromatographic identification of the intermediates

The data in Table I demonstrate that both intermediates II and III are formed faster than they are consumed in the subsequent oxidation step. Therefore, they should accumulate in sufficient concentrations to be isolated and identified on a paper chromatogram. Unfortunately, no single solvent has been found that would separate all four components (I–IV) of the reaction mixture. Therefore, we had recourse to separation in two successive steps: First, by the use of solvent C, 2-chloropurine was separated from III and the latter from a mixture of II and IV (see Table I). This mixture alone was then rechromatographed in solvent A. The reaction with XO was interrupted in this case at an early stage (after 20 min) by immersion in boiling water for 10 min. The solution was then concentrated *in vacuo* and spotted on paper. The results shown in Fig. 5 and 6 and in Table II give final evidence that both II and III

are present as intermediates. In view of the necessarily short duration of the enzymic reaction, the amount of IV present in this experiment was too small to be discovered by fluorescence in u.v. light. However, an extract of the appropriate zone on the chromatogram gave an absorption spectrum, identical with that of 2-chloro-6,8 dihydroxypurine (IV), isolated from enzymic reactions, which had proceeded to completion.

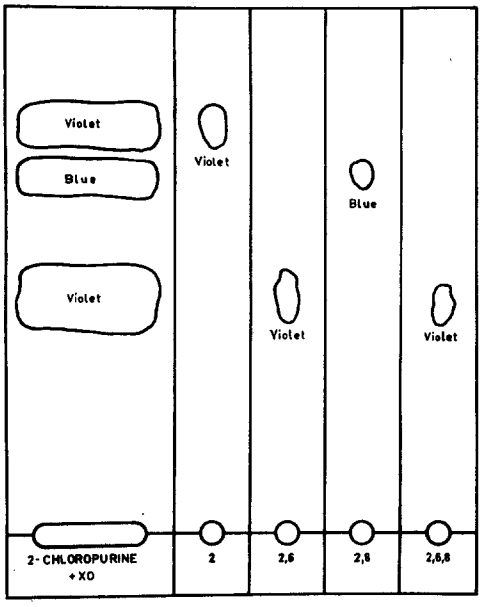


Fig. 5. Paper chromatogram of the reaction mixture, resulting from enzymic oxidation of 2-chloropurine. XO, 1:1000; substrate, $1.3 \cdot 10^{-4}$ M; pH 8.0; temperature, 28°. The reaction was stopped after 20 min. Development of the chromatogram with solvent C. Figures indicate position of substituents in the purine ring (2 always marks the chlorine atom). Note that the lowest spot on the left hand comprises a mixture of II and IV.

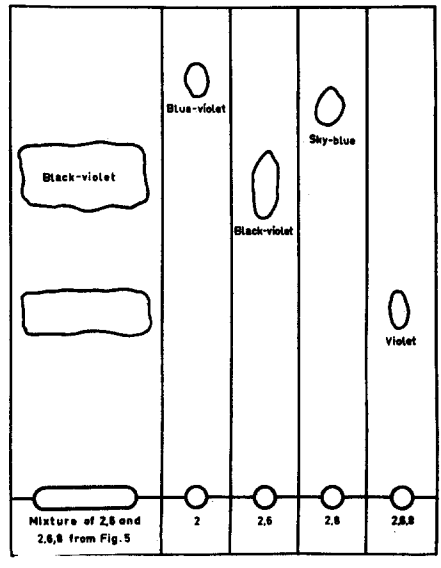
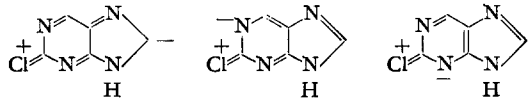


Fig. 6. Chromatographic separation in solvent A of a mixture of 2-chloro-6-hydroxypurine (II) and 2-chloro-6,8-dihydroxypurine (IV), resulting from the experiment in Fig. 5. Note that the lower spot on the left hand did not show u.v. fluorescence, but was identified by spectrophotometry of its aqueous extract.

DISCUSSION

The fact that xanthine can convert anaerobically XO into its reduced form[14] shows that the oxygen atom, introduced into the purine nucleus by the enzymic process, originates from water. Therefore, we may assume that the carbon atom with the highest positive charge will combine with a hydroxyl ion and thereby decide upon the direction of the enzymic oxidation, the ability of a specific atom to serve as reaction center being determined by the polarity of the substrate in the enzyme substrate complex[1]. Previously, it was assumed that in the first step of the reaction addition of a water molecule to a C=N group does take place, followed by transfer of two hydrogen atoms to the prosthetic group of the enzyme. However, evidence has now been obtained to support the hypothesis that direct nucleophilic substitution by OH^- may lead to the enzymic oxidation product. For the following discussion, however, this point is irrelevant.

The low reactivity of chlorine in position 2 of various purines[7-9] can be explained on the same basis as the stability of the halogen in chlorobenzene: The C–Cl bond possesses partial double bond character, due to contributions of structures such as Ia, Ib and Ic, formulated on the assumption that the tautomeric hydrogen in the imidazol ring is located mainly at the 9-position, like in purine itself[15]. Among these



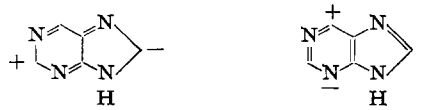
Ia Ib Ic

three resonance forms, only Ia and Ib contain the triad system $\overset{3}{N}=\overset{4}{C}-\overset{9}{N}H$, which presumably attaches itself to an appropriate receptor group in the active center of XO[1]. Now it is evident that in Ia only C-6 may be attacked by a hydroxyl ion, C-8 being incapacitated by its formal negative charge. Similarly, in Ib only C-8 may serve as center for nucleophilic attack by OH^-, C-6 no longer participating in a polarisable C=N group, but being close to the negatively charged N-1 atom.

The fact that 2-chloropurine is attacked by XO at C-6 and C-8 with practically identical rates, may now be interpreted in the following way: In the ES complex, the polarities contributed by Ia and Ib are of approximately equal importance. A hydroxyl ion, approaching the purine nucleus, does therefore have equal chances to become attached to either position 6 or 8.

The above considerations may also shed light on the behavior of purine itself towards XO. In purine, no polar substituent is present to induce special distributions of the π-electrons. *E.g.*, on account of C-8 becoming a positive center, either C-6 or C-2 must assume a negative charge (see Va and Vb). Similarly, a positive charge at C-2 will place a negative pole on C-8 (Vc). Only carbon atom 6 is able to use the intrinsic polarity of the two C=N groups in the pyrimidine ring for the electron distribution, indicated in Vd, where the opposite charges are sufficiently far apart to permit the approach of OH^- to position 6. In addition, Vd is the only resonance hybrid, in which the central double bond is undisturbed and the aromatic character of the imidazol ring preserved. This resonance form, therefore, makes the greatest contribution to the structure of purine in the activated enzyme substrate complex.

Va Vb



Vc Vd

It is evident that the "active" form Vd does not possess the characteristic triad group $\overset{3}{N}=\overset{4}{C}-\overset{9}{N}H$. This is in accord with our previous observation that methylation of either N-7 or N-9 in purine does not prevent enzymic oxidation[1].

The observations presented here invite a study of other purines, bearing a single polar substituent in various positions. These experiments will be reported in due course.

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