Oxidation of Quinazoline and Quinoxaline by Xanthine Oxidase and Aldehyde Oxidase

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Quinazoline is oxidized by xanthine oxidase initially (and rapidly) to 4-hydroxyquinazoline which subsequently is oxidized more slowly to 2,4-dihydroxyquinazoline. Both oxidative reactions are inhibited strongly by allopurinol. Quinazoline is oxidized by aldehyde oxidase to 4-hydroxyquinazoline but within a short time (3-5 minutes) the reaction ceases; the proposal that cessation of reaction is due to product inhibition is rendered untenable by our observation that 4-hydroxyquinazoline is rapidly oxidized by aldehyde oxidase to 2,4-dihydroxyquinazoline. Preincubation of aldehyde oxidase with quinazoline results in complete inhibition of the ability of the enzyme to oxidize 4-hydroxyquinazoline and the standard substrate *N*-methylnicotinamide. It appears therefore that quinazoline is able to react with aldehyde oxidase and inactivate it. Quinoxaline and 2-hydroxyquinoxaline are not oxidized by xanthine oxidase but are converted by aldehyde oxidase to 2,3-dihydroxyquinoxaline; all oxidations mediated by aldehyde oxidase are inhibited completely by menadione.

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Quinazoline (II) shares many chemical properties with pteridine (I), including susceptibility to a variety of nucleophilic addition reactions (1,2,3). Pteridine and many of its derivatives are oxidized readily by xanthine oxidase and aldehyde oxidase (4,5); quinazoline has been reported to be a substrate for milk xanthine oxidase and rabbit liver aldehyde oxidase (6) and several quinazoline derivatives have been found to be potent inhibitors of xanthine oxidase (7). Earlier studies of the enzymatic oxidation of quinazoline have not included identification of oxidation product(s), susceptibility of the reaction to inhibition by conventional inhibitory agents or estimation of the kinetics of enzyme mediated oxidation. Such studies have been carried out in our laboratory and the results of these studies are presented in this communication.

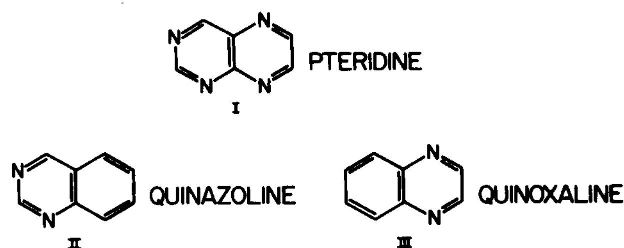

Figure 1

When quinazoline is incubated with milk xanthine oxidase a rapid alteration of the ultraviolet absorption spectrum of the reaction solution is observed; a characteristic increase in absorption in the region 300-315 nm and at 270 nm occurs during the course of the reaction. The major product formed during this rapid initial phase of the reaction exhibits spectroscopic and chromatographic properties (Table I) identical with those of 4-hydroxyquinazoline (8). The initial rapid conversion of quinazoline to 4-hydroxyquinazoline is followed by a much slower transformation of 4-hydroxyquinazoline, a product which has an absorption spectrum essentially the same as that of 2,4-dihydroxyquinazoline. When a relatively high concentration of enzyme (0.07 units/ml.) is used to catalyze the oxidation process, the initial reaction is complete in approximately 10 minutes, while the subsequent reaction requires 2-3 hours for completion. Our tentative identification of the ultimate product of quinazoline oxidation in the xanthine oxidation system is consistent with our observations and those of others (7) that 2,4-dihydroxyquinazoline undergoes no reaction when incubated for long periods of time with xanthine oxidase.

In order to establish rigorously the identity of the products observed during xanthine oxidase mediated oxidation of quinazoline we incubated relatively large amounts (100 mg.) of quinazoline (and 4-hydroxyquinazoline, the reaction intermediate) with xanthine oxidase and isolated the resulting oxidation products. Extraction of the acidified quinazoline extraction mixture after incubation for 48-96 hours with milk xanthine oxidase resulted in isolation of a crystalline solid (50% yield) which exhibited infrared and proton magnetic resonance spectra identical with those of 4-hydroxyquinazoline; residual quinazoline was detected in the reaction mixture by chromatographic analysis at this time as was the "secondary" product 2,4-dihydroxyquinazoline. Incubation of 4-hydroxyquinazoline in a "large-scale" reaction with xanthine oxidase resulted in gradual precipitation of a white solid in the reaction mixture; the infrared and pmr spectra of this product were identical to those of 2,4-dihydroxyquinazoline; the yield of product isolated in this reaction was 70%; approximately 30% of unreacted 4-hydroxyquinazoline (determined by ultraviolet spectroscopy) was present in the

Table I

Ultraviolet Spectroscopic and Chromatographic Properties of Quinazolines

Compound	Uv Maxima (*p*H)	Rf (Solvent) (a)
Quinazoline	268, 303 (7.0, 7.8) 301 (13)	0.96 (A), 0.69 (B)
Quinazoline and xanthine oxidase (initial product)	271, 303, 312 (7.0) 278, 304, 314 (13)	0.72 (A), 0.59 (B)
Quinazoline and aldehyde oxidase	271, 303, 312 (7.8)	0.72 (A)
4-Hydroxyquinazoline	271, 303, 312 (7.0, 7.8) 278, 304, 314 (13)	0.72 (A), 0.59 (B)
4-Hydroxyquinazoline and xanthine oxidase	317 (*p*H 7.0)	0.52 (B)
4-Hydroxyquinazoline and aldehyde oxidase	317 (*p*H 7.8)	0.51 (B)
2,4-Dihydroxyquinazoline	317 (*p*H 7.0, 7.8)	0.52 (B)
Quinoxaline	312 (7.0, 7.8) 312 (13)	0.62
Quinoxaline and xanthine oxidase	312 (7.0)	-----
Quinoxaline and aldehyde oxidase	310, *322* (7.8) 313, 324, 339 (13)	0.38 (B)
2-Hydroxyquinoxaline	280, 337 (7.0, 7.8) 350 (13)	0.52 (B)
2-Hydroxyquinoxaline and xanthine oxidase	280, 337 (7.0) 350 (13)	-----
2-Hydroxyquinoxaline and aldehyde oxidase	310, *322* (7.8) 313, 325, 339 (13)	0.38 (B)
2,3-Dihydroxyquinoxaline	310, *322* (7.0, 7.8) 313, 325, 339 (13)	0.40 (B)

(a) Solvent A: 2-propanol-dimethyl formamide-ammonia (65:25:10); solvent B: *p*H 7.0 phosphate buffer.

filtrate from the reaction mixture.

It is interesting to note that the initial reaction of quinazoline mediated by xanthine oxidase, that is the transformation to 4-hydroxyquinazoline, parallels the reaction observed (2) when the covalent hydrate of quinazoline is oxidized by hydrogen peroxide in acid solution; this suggests that the chemical oxidation of quinazoline in acid solution may serve as a partial model for the xanthine oxidase mediated oxidation. The conversion of 4-hydroxyquinazoline to 2,4-dihydroxyquinazoline, which we have found to be mediated by xanthine oxidase, also occurs in bacterial species as part of a degrative pathway described by Grant and Al-Najjar (9). The oxidation of both quinazoline and 4-hydroxyquinaxoline, mediated by milk xanthine oxidase, is subject to pronounced inhibition (Table II) by allopurinol, a standard inhibitor of xanthine oxidase (10).

When quinazoline is incubated with rabbit liver aldehyde oxidase a product is formed which, on the basis of spectroscopic and chromatographic properties, is identified as 4-hydroxyquinazoline. The reaction of quinazoline with aldehyde oxidase was not conventional in that oxidation of quinazoline did not progress to completion. Instead we observed an initial rapid rise in absorbancy at 270 nm but the rate of change of absorbancy subsequently decreased sharply and within a few minutes no appreciable further changes occurred in the spectrum of the reaction mixture. We first proposed that 4-hydroxyquinazoline produced by the action of aldehyde oxidase on quinazoline might be a potent inhibitor of this enzyme, and after the concentration of 4-hydroxyquinazoline produced by the enzyme reached a critical level, progressively stronger suppression of enzyme activity would be effected. This proposal appeared reasonable in view of several reports (6) of inhibition of flavoprotein oxidases by their products. We tested our proposal by incubating 4-hydroquinazoline with aldehyde oxidase and were surprised to observe an

Table II

Inhibition of Enzymatic Oxidations

System	Absorbance Change (10 minutes)
Quinazoline and xanthine oxidase	
control	0.220 (0.110) (a)
plus allopurinol	0.010 (0.004)
Quinazoline and aldehyde oxidase	
control	0.300 (b)
plus menadione	0.000
plus allopurinol	0.280
4-Hydroxyquinazoline and xanthine oxidase	
control	0.020 (c) (0.02)
plus allopurinol	0.001 (0.002)
4-Hydroxyquinazoline and aldehyde oxidase	
control	0.200 (d)
plus menadione	0.000
plus quinazoline (e)	0.000
N-Methylnicotinamide and aldehyde oxidase	
control	0.500 (f)
plus quinazoline (1 x $10^{-4}M$: no preincubation)	0.170
plus quinazoline (1 x $10^{-4}M$: 5 min. preincubation)	0.020
plus quinazoline (1 x $10^{-5}M$: 5 min. preincubation)	0.250
4-Hydroxypteridine and xanthine oxidase	
control	0.71 (g)
plus quinazoline (e)	0.60
Quinoxaline and aldehyde oxidase	
control	0.14 (h)
plus menadione	0.02
2-Hydroxyquinoxaline and aldehyde oxidase	
control	0.120 (h)
plus menadione	0.003

(a) Absorbance change at 305 nm: figures in parentheses obtained with mouse liver enzyme. (b) Absorbance change at 300 nm. (c) Absorbance change at 270 nm. (d) Absorbance change at 320 nm. (e) Quinazoline at 1 x $10^{-4}M$: 5 minute preincubation. (f) Absorbance change at 300 nm. (g) Absorbance change at 330 nm. (h) 340 nm: 60 min.

extremely rapid conversion of this compound to 2,4-dihydroxyquinazoline. Accordingly, our initial supposition, that "product inhibition" by 4-hydroxyquinazoline accounts for the incomplete conversion of quinazoline by aldehyde oxidase, became untenable. We next incubated quinazoline with aldehyde oxidase for five minutes and then added 4-hydroxyquinazoline to the reaction mixture; under these conditions no oxidation of 4-hydroxyquinazoline occurred. Therefore quinazoline, itself appears capable of inhibiting aldehyde oxidase and this inhibition is increased markedly by pre-incubation of quinazoline with the enzyme. Quinazoline also inhibits the oxidation of *N*-methyl nicotinamide catalyzed by aldehyde oxidase and again inhibition was more pronounced when quinazoline was pre-incubated with the enzyme prior to substrate addition (Table II). It has been reported (3) that quinazoline can react readily with sulfhydryl groups; it is possible that the inhibition of aldehyde oxidase by quinazoline involves interaction with sulfhydryl groups important for the catalytic functions of the enzyme. The observation that quinazoline can inhibit aldehyde oxidase complicates interpretations of the results of previous studies (6) comparing the rates of oxidation of quinazoline and other substrates by aldehyde oxidase. 2,4-Dihydroxyquinazoline was found to act neither as a substrate nor as an inhibitor of aldehyde oxidase. The oxidation of quinazoline and of 4-hydroxyquinazoline by aldehyde oxidase was completely inhibited by menadione, a potent inhibitor (11) of the oxidation of a variety of substrates by this enzyme.

It should be pointed out that Stubley and colleagues, in a recent abstract (12) describing the metabolism of nitrogen heterocycles by aldehyde oxidase and hepatic microsomal oxidases, have reported that quinazoline and 4-hydroxyquinazoline are oxidized to 4-hydroxyquinazoline and 2,4-dihydroxyquinazoline respectively by aldehyde oxidase but no mention is made of the inhibitory

effects of quinazoline.

Oxidation of pteridine derivatives proceeds in a similar manner with both milk xanthine oxidase and rat liver xanthine oxidase (5). We found that quinazoline and 4-hydroxyquinazoline are each oxidized by rat and mouse liver xanthine oxidase to the same products as observed in the milk xanthine oxidase system. We also found that xanthine oxidase from monkey liver behaves like the milk enzyme in converting quinazoline first to 4-hydroxyquinazoline with subsequent formation of the 2,4-dihydroxy derivative. Oxidation of quinazolines by mammalian xanthine oxidases was inhibited strongly by allopurinol.

It is interesting to contrast the ability of quinazolines to inhibit aldehyde oxidase with the lack of activity of pteridine as an inhibitor of this enzyme. One is tempted to suggest that the difference in the abilities of these formally related heterocyclic compounds to inhibit aldehyde oxidase is related to differences in their tendencies to undergo covalent hydration under physiological conditions. For example, quinazoline undergoes little hydration at *p*H 7.0 (2) while pteridine is significantly hydrated under comparable conditions (13); accordingly the inhibitory action of quinazoline may be associated with the high proportion of anhydrous (not covalently hydrated) molecules in neutral aqueous solution compared with pteridine.

Quinoxaline (III) an isomer of quinazoline has been found to be unreactive toward *acid-catalyzed* covalent hydration under reaction conditions in which quinazoline (II) is hydrated extensively (14). In some instances the relative susceptibilities of pteridines to acid-catalyzed covalent hydration is paralleled by their relative susceptibilities to oxidation mediated by xanthine oxidase (15). We were unable to detect oxidation of quinoxaline by xanthine oxidase from milk or mouse liver. The observation that quinoxaline undergoes neither acid-catalyzed covalent hydration nor oxidation by xanthine oxidase while quinazoline reacts readily in both situations is consistent with previous suggestions (16) that a nucleophilic reaction analogous to covalent hydration is involved in xanthine oxidase mediated reactions. When quinoxaline is incubated with aldehyde oxidase a product is obtained, which on the basis of spectroscopic and chromatographic characteristics is identified as 2,3-dihydroxyquinoxaline. The same product is observed when 2-hydroxyquinoxaline is incubated with aldehyde oxidase; oxidation of both quinoxaline and its 2-hydroxyderivative by aldehyde oxidase is inhibited completely by menadione (Table II). Stubley, *et al.*, reported (12) that quinoxaline is converted to 2-hydroxyquinoxaline by aldehyde oxidase even though they also report that the same enzyme system converts 2-hydroxyquinoxaline to 2,3-dihydroxyquinoxaline; the use by these authors of a crude source of enzyme activity (100,000 x g. supernatant fraction of a rabbit liver homogenate) complicates interpretation of their results as does their failure to report studies carried out in the presence of menadione (or an alternative selective inhibitor of aldehyde oxidase).

Investigation of the effects of alteration of quinazoline concentration of the rate of oxidation mediated by milk xanthine oxidase demonstrated that the reaction exhibits conventional kinetic behavior. A Km value of 4.2×10^{-4} M was estimated (Table III) for quinazoline as a substrate for milk xanthine oxidase; a somewhat higher apparent affinity was observed for 4-hydroxyquinazoline (Km = $7.8 \times 10^{-5} M$). A Km value of $1.56 \times 10^{-5} M$ for quinazoline in the aldehyde oxidase system has been estimated (12). We find, using oxygen as electron acceptor, a higher Km ($6.1 \times 10^{-5} M$) for quinazoline oxidation by aldehyde oxidase; the Km value which we estimated for 4-hydroxyquinazoline ($7.5 \times 10^{-5} M$) is closely similar to that for quinazoline. We found the maximum velocity of xanthine oxidase mediated oxidation of quinazoline to be 6-7 times higher than that observed with 4-hydroxyquinazoline; in the aldehyde oxidase system on the other hand the maxi-

Table III

Kinetic Studies of Enzymatic Oxidation

Substrate	Enzyme	K_m (M)	V max (moles/liter/minute)
Quinazoline	Milk xanthine oxidase	4.2×10^{-4}	9.1×10^{-5}
	Mouse liver xanthine oxidase	1.7×10^{-4}	-----
	Aldehyde oxidase	6.1×10^{-5} (1.2×10^{-4}) (a)	4.3×10^{-5}
4-Hydroxyquinazoline	Milk xanthine oxidase (a)	7.8×10^{-5}	1.4×10^{-5}
	Aldehyde oxidase	7.5×10^{-5}	6.6×10^{-5}
Quinoxaline	Aldehyde oxidase	3.6×10^{-3} (2.6×10^{-4}) (a)	1.0×10^{-5}
2-Hydroxyquinoxaline	Aldehyde oxidase	2.5×10^{-4}	2.2×10^{-6}

(a) Using ferricyanide (6) rather than oxygen as electron acceptor.

mum velocities of enzymatic oxidation were essentially the same for both quinazoline and its 4-hydroxy derivative. The *Km* value estimated for 2-hydroxyquinoxaline as a substrate for aldehyde oxidase is $2.5 \times 10^{-4} M$; the *Km* value which we estimated for quinoxaline is considerably higher ($3.6 \times 10^{-3} M$). A *Km* value of $1.6 \times 10^{-4} M$ for quinoxaline in the aldehyde oxidase system was reported by Stubley, *et al.* (12); it is not clear from their communication whether this value was obtained directly (oxygen gas as electron acceptor) or by measuring ferricyanide reduction; with ferricyanide (6) as electron acceptor we find a *Km* value of $2.6 \times 10^{-4} M$. The discrepancies between *Km* values observed for quinazoline and quinoxaline, using direct and indirect assay procedures, indicates that comparisons of kinetic properties obtained by the different assay systems may frequently be invalid.

EXPERIMENTAL

Ultraviolet spectroscopic measurements were made using either a Perkin-Elmer Model 202 recording spectrophotometer or a Beckman Model 25 spectrophotometric system. All kinetic studies were performed at 37° using the Beckman system. Proton magnetic resonance spectra were obtained in deuterated dimethyl sulfoxide or in deuterium oxide to which 20% sodium deuteroxide (2 drops/ml.) was added to facilitate solution of hydroxy derivatives; sodium trimethylsilylpropane sulfonic acid was employed as an internal standard and measurements were made using a Perkin-Elmer Model R-12 instrument. Infrared spectra were determined in nujol mulls with a Perkin-Elmer Model 137 spectrophotometer. Thin layer chromatographic studies were performed on either Avicel or MN-cellulose plates (Analtech, Newark, Del.) using either isopropanol-ammonia-dimethylformamide (65-10-25), or phosphate buffer (0.1*M*; *p*H 7.0) as solvents. Compounds were detected by visualization at either 256 or 360 nm. Milk xanthine oxidase was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio and Sigma Chemical Co., St. Louis, Missouri; catalase was obtained from Worthington Biochemical Co., Freehold, New Jersey. Rabbit liver aldehyde oxidase was obtained by the procedure described by Wolpert and her colleagues (17); xanthine oxidases from rat liver, mouse liver and monkey liver (Pel-Freez, Inc., Rogers, Arkansas) were partially purified by the method outlined by Kielley (18). All quinazoline derivatives, quinoxaline and 4-hydroxypyrazolo[3,4-*d*]pyrimidine (allopurinol) were purchased from Aldrich Chemical Co., Milwaukee, Wisconsin. Published procedures were used to synthesize 2-hydroxyquinoxaline (19) and 2,3-dihydroxyquinoxaline (20; peroxide oxidation method); these compounds also may be obtained from Aldrich. Studies with xanthine oxidase were carried out in phosphate buffer (0.1*M*; *p*H 7.0); studies with aldehyde oxidase were performed in Tris buffer (0.05*M*; *p*H 7.8; containing 0.005% EDTA). Assays of enzymatic oxidation were done with condidate substrates at a concentration of $1 \times 10^{-4} M$. For xanthine oxidase sufficient enzyme was added to produce an absorbance change of 0.50 over 10 minutes with hypoxanthine ($1 \times 10^{-4} M$) as substrate; for aldehyde oxidase sufficient enzyme was added to produce an absorbance change of 0.50 over 10 minutes with *N*-methyl nicotinamide ($3 \times 10^{-3} M$) as substrate. Catalase (150 units/ml.) was added routinely to the aldehyde oxidase system to protect against enzyme degradation resulting from the action of hydrogen peroxide produced during the enzymatic reaction. Inhibition studies were carried out after preincubation of the appropriate enzyme system with either allopurinol ($1 \times 10^{-4} M$) or menadione ($1 \times 10^{-5} M$) for 5 minutes prior to addition of substrate. Kinetic constants were obtained from measurements made during the initial linear portion of absorbance time plots for the enzymatic oxidations.

To investigate reactions on a "large" scale quinazoline or 4-hydroxyquinazoline (0.10 g.) and xanthine oxidase (2 units; 1 unit defined as that amount of enzyme which produces 1 μmole of uric acid per minute from hypoxanthine) were added to phosphate buffer (30 ml.; *p*H 7.0) and the resulting solutions set aside in air for 2 to 7 days. The ultraviolet absorption spectrum of aliquots of the given reaction mixture was recorded twice daily during the incubation period and the reactions were terminated when the spectroscopic properties of the reaction mixture remained essentially constant. The quinazoline reaction mixture was adjusted carefully to *p*H 4.0 with glacial acetic acid and the acidified solution was extracted twice with ethyl acetate (100 ml.). Examination of the ultraviolet absorption spectrum of the aqueous solution, following extraction, indicated virtually complete extraction of quinazolines into the organic phase. The ethyl acetate extract was evaporated to dryness under reduced pressure; the residue was triturated with ethanol (10 ml.) and the mixture was filtered (gravity); the resulting filtrate was evaporated to dryness, the residue suspended in ligroin (15 ml.) and the suspension filtered (suction). The resulting white crystalline solid (50-60 mg.; approximately 50%) on the basis of infrared, proton magnetic resonance and ultraviolet spectroscopy was judged identical with an authentic sample of 4-hydroxyquinazoline.

The 4-hydroxyquinazoline reaction mixture was filtered with suction to yield a solid (65 mg.) which proved identical to 2,4-dihydroxyquinazoline on the basis of comparative spectroscopic properties. The filtrate was found to contain a large amount (30 mg.; estimated by ultraviolet spectroscopy) of unreacted 4-hydroxyquinazoline. To eliminate the possibility that non-enzymatic alteration of quinazoline or 4-hydroxyquinazoline contributed to the changes observed when these compounds are incubated for long periods with xanthine oxidase, both compounds (0.01 mg.) were set aside for 5 days in phosphate buffer (3.0 ml.) and chromatographic and spectroscopic analysis of the solutions were performed daily; neither quinazoline nor its 4-hydroxy derivative were altered under these conditions.

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