Oxidation of Selected Pteridine Derivatives by Mammalian Liver Xanthine Oxidase and Aldehyde Oxidase

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Abstract □ Considerable information is available concerning the oxidation of pteridine derivatives by bovine milk xanthine oxidase, but few investigations have been carried out on the oxidation of such compounds by mammalian liver xanthine oxidase and the related aldehyde oxidase. Xanthine oxidase, obtained from rat liver, oxidizes a variety of substituted amino- and hydroxypteridines in a manner identical to that previously observed for milk xanthine oxidase. For example, 2-aminopteridine and its 4- and 7-hydroxy derivatives were oxidized efficiently to 2-amino-4,7-dihydroxypteridine (isoxanthopterin) by the rat liver enzyme, and 4-aminopteridine and its 2- and 7-hydroxy derivatives were oxidized to 4-amino-2,7-dihydroxypteridine. 4-Hydroxypteridine and the isomeric 2- and 7-hydroxypteridines were oxidized by rat liver xanthine oxidase to 2,4,7-trihydroxypteridine. Rabbit liver aldehyde oxidase, but not rat liver xanthine oxidase, was able to catalyze the oxidation in position 7 of 2,4-diaminopteridine and its 6-methyl and 6-hydroxymethyl derivatives. 2-Aminopteridine and 4-aminopteridine were both oxidized to the corresponding 7-hydroxy derivatives in the aldehyde oxidase system; 2-amino-4-hydroxypteridine appeared to be a minor product in the oxidation of 2-aminopteridine by rabbit liver aldehyde oxidase. Both aldehyde oxidase and xanthine oxidase were able to catalyze the oxidation of 2-amino-6,7-disubstituted pteridines to the corresponding 4-hydroxy derivatives; 4-hydroxy-6,7-disubstituted pteridines were oxidized in position 2 by both enzymes. 4-Amino-6,7-disubstituted pteridines were not oxidized by either enzyme. 2-Amino-4-methylpteridine was oxidized in position 7 by aldehyde oxidase but was not an effective substrate for xanthine oxidase; 2-hydroxypteridine and 7-hydroxypteridine were not oxidized to a detectable extent by aldehyde oxidase. All oxidations mediated by xanthine oxidase were strongly inhibited by allopurinol (4-hydroxypyrazolo[3,4-*d*]pyrimidine), and all oxidations mediated by aldehyde oxidase were inhibited by menadione (2-methyl-1,4-naphthoquinone). Rat liver xanthine oxidase and, to a lesser extent, rabbit liver aldehyde oxidase were inhibited by 4-chloro-6,7-dimethylpteridine; 2-amino-3-pyrazinecarboxylic acid inhibited xanthine oxidase but not aldehyde oxidase. The oxidations of 2- and 4-aminopteridines by aldehyde oxidase resulted in concomitant reduction of cytochrome c.

Keyphrases □ Pteridine derivatives—oxidation by mammalian liver xanthine oxidase and aldehyde oxidase □ Xanthine oxidase—mammalian liver, oxidation of pteridine derivatives □ Aldehyde oxidase—mammalian liver, oxidation of pteridine derivatives □ Enzymes—mammalian liver xanthine oxidase and aldehyde oxidase, oxidation of pteridine derivatives □ Oxidation—pteridine derivatives by mammalian liver xanthine oxidase and aldehyde oxidase

Xanthine oxidase (xanthine:oxygen oxidoreductase, EC 1.2.3.2) and aldehyde oxidase (aldehyde:oxygen oxidoreductase, EC 1.2.3.1) have a number of common characteristics (1), *e.g.*, similar molecular weights, the ability to utilize a variety of electron acceptors, and the presence of a nonheme iron, molybdenum, and flavin adenine dinucleotide. The presence of a quinone of the coenzyme Q type has been demonstrated (2) in aldehyde oxidase but not in xanthine oxidase; however, recent studies (3) called into question the significance of a coenzyme Q type of factor in the function of aldehyde oxidase.

Both enzymes are found in mammalian liver and both are capable of oxidizing various substrates including heterocyclic compounds of the purine (4), pteridine, and azapteridine (5–11) types and aldehydes (12, 13). Both enzymes are capable of oxidizing important pharmacological agents; for example, xanthine oxidase oxidizes the antineoplastic agent mercaptopurine (14), and aldehyde oxidase oxidizes methotrexate (15), an agent used extensively in antineoplastic therapy, and the immunosuppressive agent azathioprine (16). The xanthine oxidase system also has been demonstrated (17) to be capable of participating indirectly in oxidative reactions (*via* generation of superoxide radicals), and both xanthine oxidase and aldehyde oxidase mediate reductive reactions of the nitro group of important drugs (18–20).

Certain pteridine derivatives are used clinically (21–23) and have been implicated as important participants in biochemical functions (24–27). Because of these considerations, the chemistry and biochemistry of pteridine derivatives and the behavior of pteridines were investigated in several enzyme systems.

Previously (7), the behavior of aminopteridines in the milk xanthine oxidase system was described. It was of interest to determine whether the same reaction patterns observed in oxidations catalyzed by the milk enzyme would be observed with rat liver xanthine oxidase and whether the oxidation patterns determined for xanthine oxidase were similar to those observed with the analogous enzyme, aldehyde oxidase.

Since studies of the abilities of heterocyclic compounds to serve as substrates and/or inhibitors of xanthine oxidase frequently are carried out with the enzyme obtained from milk, it was important to investigate whether the substrate specificity for pteridine oxidation mediated by liver xanthine oxidase was the same as that observed with the "standard" milk enzyme and whether the same products were formed in the two enzyme systems. Information about the relative susceptibilities of pteridine derivatives to liver xanthine oxidase and aldehyde oxidase *in vitro* can be of value in predicting patterns of oxidation *in vivo* and also in the design of new inhibitors of these enzymes.

This report presents the results of studies carried out to ascertain the behavior of pteridines in the xanthine oxidase system obtained from rat liver and the aldehyde oxidase system obtained from rabbit liver.

EXPERIMENTAL

The oxidation of pteridine derivatives by xanthine oxidase and aldehyde oxidase was investigated by spectrophotometric and chromatographic techniques. For spectrophotometric studies, reaction solutions were scanned in the 270–390-nm region using a recording spectrophotometer[1] equipped with a thermostated sample chamber at 34 ± 1°. Rat liver xanthine oxidase was prepared by the method of Kielley (28) up to the fifth ammonium sulfate step. Aldehyde oxi-

[1] Perkin-Elmer model 202.

dase was prepared from rabbit liver using a modification of the method of Rajagopalan *et al.* (2). In agreement with recent studies (18), aldehyde oxidase that was substantially free from xanthine oxidase activity was obtained using appropriate ammonium sulfate fractionations without chromatographic procedures. Bovine milk xanthine oxidase was a commercial product[2].

Studies with xanthine oxidase were carried out using a trometh-amine buffer system (0.05 *M*, pH 7.8, containing 0.005% ethylenediaminetetraacetic acid), the pteridine derivative (1×10^{-4} *M*), and sufficient xanthine oxidase to give an absorbance change of 0.50 unit/10 min at 290 nm, evaluated with hypoxanthine (1×10^{-4} *M*) as the standard substrate. Catalase[2] (151 units/ml) was added routinely to the xanthine oxidase system even though, as found previously (8) for milk xanthine oxidase, catalase did not affect the rate of pteridine oxidation by liver xanthine oxidase. Catalase was added to the xanthine oxidase system simply to ensure that this system resembled as closely as possible the aldehyde oxidase system described here.

All pteridine derivative substrates for rat liver xanthine oxidase were examined in a system containing a high concentration (1×10^{-4} *M*) of allopurinol (4-hydroxypyrazolo[3,4-*d*]pyrimidine)[3] to establish the susceptibility of the xanthine oxidase-mediated reaction to this inhibitor. The classification of compounds as ineffective substrates for xanthine oxidase was based on the failure to demonstrate a detectable alteration in the absorption spectrum of the compound over 3–4 hr in the presence of the enzyme.

Essentially the same procedure as already outlined was used for spectrophotometric studies with rabbit liver aldehyde oxidase; the composition of the reaction solution was identical except that xanthine oxidase was replaced by aldehyde oxidase in an amount sufficient to produce an absorbance change of 0.50 unit/10 min at 300 nm, using the standard substrate N^1-methylnicotinamide (3×10^{-3} *M*). Catalase addition was required for maximal activity with aldehyde oxidase. For inhibition studies with aldehyde oxidase, menadione (2-methyl-1,4-naphthoquinone[4], 1×10^{-5} *M*) was added to the reaction vessel.

All but two pteridine derivatives used were synthesized by published procedures. 2-Amino-4-hydroxy-6,7-cyclohexenopteridine was prepared in the following manner. 6-Hydroxy-2,4,5-triaminopyrimidine sulfate[3] (0.40 g) was suspended in water (10 ml), and sodium bicarbonate was added carefully to the mixture until pH 7 was attained. Cyclohexane-1,2-dione[3] (0.20 g) and ethanol (1 ml) then were added, and the mixture was refluxed on a water bath for 4 hr. The precipitate that formed upon chilling the reaction mixture was isolated by suction filtration and washed with successive portions of water and ethanol. The UV absorption characteristics of the compound were identical to material obtained by acid hydrolysis of 2,4-diamino-6,7-cyclohexenopteridine[5], and the substance migrated as a single spot in several chromatographic systems.

The identities of reaction products formed when various pteridines were incubated with xanthine oxidase or aldehyde oxidase, initially assigned by spectroscopic methods, were confirmed by TLC. Reaction solutions identical with those already described, except that the pteridine concentration was 3×10^{-4} *M*, were incubated overnight at 37° to ensure complete conversion of the substrate to hydroxylated derivatives. The reaction solutions were heated at 90° for 5 min to inactivate the enzymes, and the mixtures were centrifuged to remove precipitated protein.

Aliquots (10 and 20 μl) of the supernatant solutions were applied to analytical TLC plates[6] along with pertinent standard pteridines, and the chromatograms were developed in the appropriate solvent systems. Pteridine spots were visualized using both short (256 nm) and long (360 nm) wavelength UV light in a chromatogram-viewing cabinet equipped with a transilluminator[7]. Identification of reaction products was made on the basis of comparison of the chromatographic mobility and fluorescence properties with those of standard compounds.

The reduction of cytochrome c was investigated by adding cytochrome c[8] (1×10^{-5} *M*) to the described aldehyde oxidase reaction solutions and observing the appearance of the characteristic absorption maximum of reduced cytochrome c at 550 nm.

RESULTS

Pteridine (7) itself was oxidized readily by both rat liver xanthine oxidase and by aldehyde oxidase; however, the pattern of spectral change observed during the reaction was different for the two enzymes. For example, while both reactions were characterized by the appearance of a new absorption maximum at 328 nm, the intensity of the new peak in the xanthine oxidase reaction was approximately twice that for the aldehyde oxidase reaction, and a prominent shoulder was observed at 338 nm in the xanthine oxidase reaction but not in the aldehyde oxidase reaction.

Comparison of the UV absorption maxima observed for the product of the xanthine oxidase-catalyzed reaction with those of 2,4,7-trihydroxypteridine (7) (Table I) suggested that pteridine is converted, in the rat liver system, to the latter compound. TLC analysis also indicated that the product of this reaction was 2,4,7-trihydroxypteridine. On the other hand, the aldehyde oxidase-mediated conversion of pteridine did not yield the trihydroxylated derivative but gave a product identified as 2,4-dihydroxypteridine (7) (lumazine) on the basis of its spectroscopic properties. Chromatographic analysis of the aldehyde oxidase mixture revealed a major spot corresponding to 2,4-dihydroxypteridine and a minor spot corresponding to 4-hydroxypteridine (7). The presence of the 4-hydroxypteridine spot suggests that this compound may be an intermediate in the aldehyde oxidase-catalyzed oxidation of pteridine.

When these studies were completed on the oxidation of pteridine by aldehyde oxidase, a report by Krenitsky *et al.* (5) described their studies of the same reaction. Our results are in agreement with their studies with regard to the formation of 2,4-dihydroxypteridine as the major ultimate product of the reaction of pteridine with aldehyde oxidase.

When 2-hydroxy- (7), 4-hydroxy-, 7-hydroxy- (7), and 2,4-dihydroxypteridines were incubated with xanthine oxidase, the final absorption spectrum of each reaction mixture was identical with that of 2,4,7-trihydroxypteridine, and the chromatographic properties of the products also were those of the trihydroxy derivative. It was reported previously (9) that 4-hydroxypteridine is oxidized by rabbit liver aldehyde oxidase only in position 2, and Krenitsky *et al.* (5) confirmed this observation.

The spectroscopic and chromatographic properties of the product of the reaction of 4-hydroxypteridine with aldehyde oxidase are summarized in Table I. The 6,7-dimethyl derivative (29) and the 6,7-cyclohexeno derivative (30) of 4-hydroxypteridine were oxidized in position 2 by aldehyde oxidase, and the same products (30, 31) were obtained when these substrates were treated with rat liver xanthine oxidase. Incubation of 2-hydroxy-, 2,4-dihydroxy-, or 7-hydroxypteridine with aldehyde oxidase did not result in detectable spectroscopic changes over a 4-hr period.

Aminopteridines—2-Amino-, 2-amino-4-hydroxy-, and 2-amino-7-hydroxypteridines (8) were oxidized readily by rat liver xanthine oxidase to a common product, exhibiting spectral maxima at 282 and 333 nm. This product was identified as 2-amino-4,7-dihydroxypteridine (isoxanthopterin) (8) on the basis of its spectral characteristics and its behavior on TLC. Neither 2-amino-4-hydroxypteridine nor its 7-hydroxy isomer was oxidized by aldehyde oxidase. Indeed, both substances were products of the reaction of 2-aminopteridine with this enzyme; chromatographic analysis of the reaction mixture as well as spectroscopic studies indicated that oxidation of 2-aminopteridines by aldehyde oxidase occurs mainly in position 7. Oxidation of 2-amino-6,7-dimethylpteridine (8) and its 6,7-diphenyl and 6,7-cyclohexeno derivatives (32) was mediated by both rat liver xanthine oxidase and rabbit liver aldehyde oxidase; in all cases, oxidation was observed at position 4.

2,4-Diaminopteridine (33) and 2-amino-4-methylpteridine (8) are not oxidized by milk xanthine oxidase (8, 34); these compounds also do not serve as substrates for hepatic xanthine oxidase. On the other hand, both 2,4-diaminopteridine and 2-amino-4-methylpteridine were oxidized by aldehyde oxidase to products identified spectroscopically as the respective 7-hydroxy derivatives[9] (35). 2,4-Diamino-6-meth-

[2] Worthington Biochemical Co., Freehold, N.J.
[3] Aldrich Chemical Co., Milwaukee, Wis.
[4] Merck and Co., Rahway, N.J.
[5] Provided by Professor E. C. Taylor, Princeton University.
[6] MN cellulose and Avicel, Analtech, Newark, Del.
[7] Ultraviolet Products, Inc., San Gabriel, Calif.
[8] Sigma Chemical Co., St. Louis, Mo.

[9] The assignment of the 7-hydroxy structure to the product of oxidation of 2-amino-4-methylpteridine was done on the basis of the similarity of the spectral characteristics of this product to those of 2-amino-7-hydroxypteridine.

Table I—Spectroscopic and Chromatographic Properties of Pteridines and Pteridine Products of Oxidation by Rat Liver Xanthine Oxidase and Rabbit Liver Aldehyde Oxidase[a]

Pteridine Derivative	UV Maxima	R_f	Solvent System
Pteridine	300, 310	Streak	A
2-Hydroxypteridine	308	0.57	A
4-Hydroxypteridine	328	0.39	A
7-Hydroxypteridine	261, 328	0.65	A
2,4-Dihydroxypteridine	270, 328	0.51	A
2,4,7-Trihydroxypteridine	275, 328, 338[b]	0.22	A
Product of pteridine and its 2-, 4-, and 7-hydroxy- and 2,4-dihydroxy derivatives and xanthine oxidase	275, 328, 338[b]	0.22	A
Product of pteridine and 4-hydroxypteridine and aldehyde oxidase	270, 328	0.51	A
2-Aminopteridine	370	0.40	B
2-Amino-4-hydroxypteridine	270, 346	0.19	B
2-Amino-7-hydroxypteridine	270, 341, 353[b]	0.30	B
2-Amino-4,7-dihydroxypteridine	282, 333	0.01	B
Product of 2-aminopteridine and its 4- and 7-hydroxy derivatives and xanthine oxidase	282, 333	0.01	B
Product of 2-aminopteridine and aldehyde oxidase	270, 341, 353[b]	0.30, 0.19[c]	B
2-Amino-6,7-dimethylpteridine	368	0.72	B
2-Amino-4-hydroxy-6,7-dimethylpteridine	346	0.35	B
Product with xanthine and aldehyde oxidase	346	0.35	B
2-Amino-6,7-diphenylpteridine	281[d]	0.92	B
2-Amino-4-hydroxy-6,7-diphenylpteridine	301[d]	0.44	B
Product with xanthine and aldehyde oxidase	301[d]	0.44	B
2-Amino-6,7-cyclohexenopteridine	265, 372	0.55	B
2-Amino-4-hydroxy-6,7-cyclohexenopteridine	275, 350	0.34	B
Product with xanthine and aldehyde oxidase	275, 350	0.34	B
2,4-Diaminopteridine	365	0.17	D
2,4-Diamino-7-hydroxypteridine	353	0.22	D
Product with aldehyde oxidase	353	0.22	D
2,4-Diamino-6-methylpteridine	367	0.29	B
2,4-Diamino-6-methyl-7-hydroxypteridine	353	0.17	B
Product with aldehyde oxidase	353	0.17	B
2,4-Diamino-6-hydroxymethylpteridine	371	0.29	B
Product with aldehyde oxidase	350	0.14	B
2-Amino-4-methylpteridine	367	—	B
Product with aldehyde oxidase	345, 353[b]	—	B
4-Aminopteridine	337	0.48	B
4-Amino-2-hydroxypteridine	285, 337, 351[b]	0.18	B
4-Amino-7-hydroxypteridine	332, 344	0.33	B
4-Amino-2,7-dihydroxypteridine	276, 335, 350[b]	0.24	B
Product of 4-aminopteridine and its 2- and 7-hydroxy derivatives with xanthine oxidase	276, 335, 350[b]	0.24	B
Product of 4-aminopteridine and aldehyde oxidase	332, 344	0.33	B
4-Hydroxy-6,7-dimethylpteridine	275, 316	0.39	B
2,4-Dihydroxy-6,7-dimethylpteridine	270, 332	0.31	B
Product with xanthine and aldehyde oxidase	270, 332	0.31	B
4-Hydroxy-6,7-cyclohexenopteridine	321	0.26	C
2,4-Dihydroxy-6,7-cyclohexenopteridine	340	0.24	C
Product with xanthine and aldehyde oxidase	340	0.24	C

[a] Spectra were recorded in the 270–390-nm range in tromethamine buffer (0.05 *M*, pH 7.8) containing enzyme as described under *Experimental*. Pteridine derivatives were spotted 2.5 cm from the bottom of prepared plates, the spots were dried with warm forced air, the solvent was allowed to ascend the plate for a distance of 15 cm, and the spots were visualized with UV light. TLC solvent systems used were the following: A, 2-methyl-1-propanol–dimethylformamide–water (65:25:10); B, ethanol–ammonia–water (80:10:10); C, 2-propanol–dimethylformamide–ammonia (65:25:10); and D, water. [b] Shoulder. [c] Minor component. [d] Maximum observed also >400 nm.

ylpteridine and its 6-hydroxymethyl analog (36) also were oxidized by aldehyde oxidase to products formulated as 7-hydroxy derivatives (37) based on their spectroscopic properties. 2,4-Diamino-6-hydroxymethylpteridine was not oxidized by liver xanthine oxidase.

4-Aminopteridine, as well as its 2-hydroxy and 7-hydroxy derivatives (8), were oxidized by rat liver xanthine oxidase to 4-amino-2,7-dihydroxypteridine (8). 4-Aminopteridine was oxidized by aldehyde oxidase to a product exhibiting spectroscopic and chromatographic properties identical with those of 4-amino-7-hydroxypteridine. Neither 4-amino-7-hydroxypteridine nor its 2-hydroxy isomer was an effective substrate for aldehyde oxidase under the reaction conditions employed. It was reported previously (8, 30) that 4-amino-6,7-dimethylpteridine and 4-amino-6,7-cyclohexenopteridine were not oxidized by bovine milk oxidase, and these compounds were not oxidized by either rat liver xanthine oxidase or by aldehyde oxidase. When 4-amino-6,7-diphenylpteridine (32) was incubated with aldehyde oxidase, no spectroscopic evidence was obtained for oxidation.

Inhibition Studies—The oxidation of various substrates, including pteridines (8), by milk xanthine oxidase is susceptible to inhibition by allopurinol. All oxidations of pteridine derivatives catalyzed by rat liver xanthine oxidase are inhibited strongly by this agent. Thus, in the presence of a relatively high concentration (1×10^{-4} *M*) of allopurinol in the various reaction mixtures, inhibition of oxidation of more than 95% was observed. Menadione is a potent inhibitor of many oxidations catalyzed by aldehyde oxidase (38). Inclusion of menadione (1×10^{-5} *M*) in reaction mixtures containing pteridine derivatives and aldehyde oxidase resulted in virtually complete (greater than 90%) inhibition of pteridine oxidation. Thus, oxidation of pteridine derivatives by both mammalian liver enzyme systems studied exhibited conventional sensitivity to standard inhibitory agents.

It was observed previously that 2-amino-3-pyrazinecarboxylic acid (30), a substance that can be formed by chemical degradation (39) of 4-hydroxypteridine, is a moderately potent inhibitor of milk xanthine oxidase. 2-Amino-3-pyrazinecarboxylic acid also inhibited rat liver xanthine oxidase; no significant inhibition by 2-amino-3-pyrazinecarboxylic acid was observed (at 1×10^{-4} *M*) against aldehyde oxidase. No evidence for detectable oxidation of this pyrazine derivative was obtained in either enzyme system.

4-Chloro-6,7-dimethylpteridine was found (30) to inhibit milk

xanthine oxidase, and an apparent K_I value of 5×10^{-7} M was estimated for it. This chloropteridine did inhibit rat liver xanthine oxidase to an extent comparable with that observed with the enzyme obtained from milk. 4-Chloro-6,7-dimethylpteridine inhibited aldehyde oxidase, but the degree of inhibition was modest. For example, the chloropteridine (1×10^{-4} M) inhibited the oxidation of N^1-methylnicotinamide by only 30% whereas the same concentration of chloropteridine completely inhibited the oxidation of hypoxanthine by both milk and rat liver xanthine oxidase.

When investigating the effect of 4-chloro-6,7-dimethylpteridine on the hepatic oxidases, the use of tromethamine buffer was unsuitable. Therefore, a phosphate buffer was employed in these inhibition studies. 4-Chloro-6,7-dimethylpteridine underwent a transformation in tromethamine buffer, as evidenced by a gradual disappearance of the spectral maxima at 306 and 318 nm with the concomitant appearance of a new peak at 346 nm. Daley and Christensen (40) reported that 4-chloro-6,7-dimethylpteridine is very susceptible to nucleophilic displacement reactions at position 4 and can be readily hydrolyzed to the 4-hydroxy derivative and aminated to the 4-amino derivative. The final absorption spectrum of the product of the reaction of 4-chloro-6,7-dimethylpteridine in the tromethamine system resembled the spectrum of 4-amino-6,7-dimethylpteridine, suggesting that a product is formed in this buffer by attack of the pteridine nucleus by the amino function of tromethamine.

Cytochrome c Reduction—Both milk xanthine oxidase and aldehyde oxidase mediate a concomitant reduction of cytochrome c (2, 41) when these enzymes catalyze oxidation of a suitable substrate. Oxidations of 2-amino- and 4-aminopteridines in the aldehyde oxidase system were accompanied by reduction of cytochrome c, as evidenced by the appearance of a characteristic peak at 550 nm. The rates of appearance of reduced cytochrome c during the oxidation of the two substrates correlated well with the rate of oxidation of the substrates determined in the absence of cytochrome c.

DISCUSSION

Various pteridine derivatives are oxidized in an identical manner by rat liver xanthine oxidase and milk xanthine oxidase. Pteridines found previously to be effective substrates for the milk enzyme were found to be good substrates for the liver enzyme, and pteridines that were not oxidized by the milk enzyme also proved to be ineffective substrates for the rat liver enzyme. The relative rates of oxidation of pteridine substrates by rat liver xanthine oxidase paralleled the rates observed with the milk enzyme, and allopurinol exhibited approximately the same activity as an inhibitor of pteridine oxidation catalyzed by xanthine oxidase from either source.

The results demonstrate that pteridine oxidation reactions are closely similar whether catalyzed by milk xanthine oxidase or catalyzed by rat liver xanthine oxidase. The observations concerning the correspondence of characteristics of pteridine oxidation carried out by both enzymes parallel observations made by Bergmann and Dikstein (4) concerning purine oxidation by xanthine oxidase obtained from milk and from mammalian liver. However, at least one purine substrate appears to exhibit markedly different susceptibilities (42) to xanthine oxidase from bovine milk and that from mammalian liver.

Although aldehyde oxidase resembles xanthine oxidase, the two enzymes do not share a uniform pattern of substrate specificity. 4-Amino-6,7-dimethylpteridine and 4-amino-6,7-cyclohexenopteridine are substrates for neither aldehyde oxidase nor rat liver xanthine oxidase; this observation is in agreement with previously reported studies which described the inability of milk xanthine oxidase to oxidize these compounds. Both rat liver xanthine oxidase and rabbit liver aldehyde oxidase catalyzed the oxidation of various pteridines, resulting in the introduction of hydroxyl functions at positions 2, 4, and 7 of the pteridine ring. However, rather large differences were noted in the abilities of the two enzymes to oxidize several substrates. For example, 2,4-dihydroxypteridine was readily oxidized by the rat liver xanthine oxidase but not altered by treatment with aldehyde oxidase under comparable conditions. Similarly, 2-hydroxypteridine and 7-hydroxypteridine were oxidized by xanthine oxidase but not by aldehyde oxidase; the 4-hydroxy isomer was oxidized by both enzymes.

Presumably, hydroxyl substitution at position 2 or 7 but not at position 4 inhibits effective interaction of the pteridine ring with the catalytic site of aldehyde oxidase. Observations concerning the oxidation of pteridine at positions 2 and 4 catalyzed by aldehyde oxidase and at positions 2, 4, and 7 catalyzed by rat liver xanthine oxidase confirm those findings of Krenitsky *et al.* (5). They studied pteridine oxidation in the rabbit liver aldehyde oxidase and milk xanthine oxidase systems. Chromatographic evidence was obtained suggesting that the aldehyde oxidase-mediated oxidation of pteridine to 2,4-dihydroxypteridine proceeds through the intermediacy of 4-hydroxypteridine, and this evidence is consistent with the finding that 4-hydroxypteridine is converted to 2,4-dihydroxypteridine when incubated with aldehyde oxidase but that 2-hydroxypteridine is not oxidized in this system.

2-Amino- and 4-aminopteridines are converted to monohydroxy derivatives by aldehyde oxidase, whereas xanthine oxidase oxidizes these compounds in two positions; this observation parallels that made concerning the degree of hydroxylation produced by the two enzymes with 4-hydroxypteridine and other heterocyclic substrates as well. Xanthine oxidase can oxidize readily pteridine derivatives such as 2-amino-4-hydroxypteridine, 2-amino-7-hydroxypteridine, and 2,4-dihydroxypteridine, while aldehyde oxidase is unable to oxidize these substrates effectively. Such observations may indicate that there are subtle differences in the binding sites of the two enzymes for various substrates and/or inhibitors.

It is of interest in this regard that the 4,6-dihydroxy derivative of pyrazolo[3,4-*d*]pyrimidine is the "active metabolite" involved in the inhibition of xanthine oxidase by allopurinol and is bound (43) extremely effectively to this enzyme, whereas this compound is not a useful inhibitor of aldehyde oxidase. Thus, the dihydroxypteridine is a good substrate for xanthine oxidase and the dihydroxypyrazolopyrimidine is an excellent inhibitor of this enzyme, whereas the dihydroxypteridine is not a substrate and the dihydroxypyrazolopyrimidine is not an inhibitor of aldehyde oxidase. While both 4-aminopteridine and 2-aminopteridine were oxidized in position 7 by aldehyde oxidase, only the 2-amino derivative was found to undergo oxidation (albeit to a minor extent) in the pyrimidine portion of the molecule. The ability of 2-aminopteridine, but not its 4-amino isomer, to undergo oxidation in the pyrimidine portion when incubated with aldehyde oxidase might be related to the observation (44) that 2-aminopteridine, but not 4-aminopteridine, readily undergoes nucleophilic addition reactions (*e.g.*, covalent hydration) under appropriate conditions.

Introduction of substituents (*e.g.*, methyl groups or a cyclohexane ring) at positions 6 and 7 of the 4-aminopteridine nucleus was associated with a loss of substrate activity in both the xanthine oxidase and aldehyde oxidase systems, while the same substituents in the 2-aminopteridine nucleus did not result in a loss of substrate activity. These observations may, of course, simply indicate that the 2-position of the 6,7-disubstituted 4-aminopteridines does not effectively interact with the catalytic site of these flavoproteins, whereas the 4-position in the isomeric compounds is capable of effective interaction.

The antineoplastic agent methotrexate is a 2,4-diaminopteridine to which is attached, at position 6, a *p*-methylaminobenzoylglutamic acid function; methotrexate is oxidized readily by rabbit liver aldehyde oxidase *in vitro*, and the extent to which this agent is hydroxylated *in vivo* has been correlated with the level of aldehyde oxidase present in the liver of several mammalian species (15, 45). It was of interest to determine whether the large 6-substituent was essential for the oxidation of the 2,4-diaminopteridine nucleus by aldehyde oxidase. 2,4-Diaminopteridine and its 6-methyl and 6-hydroxymethyl derivatives were oxidized in position 7 by aldehyde oxidase; accordingly, the large *p*-aminobenzoylglutamic acid substituent is not required for oxidation of the diaminopteridine nucleus to occur. It should be stressed, however, that recent studies in this laboratory indicated that methotrexate is oxidized much more rapidly under comparable conditions than are the simpler 2,4-diaminopteridines.

The 2,4-diaminopteridine derivatives and 2-amino-4-methylpteridine proved to be unique among the pteridines investigated in that they were oxidized by aldehyde oxidase but not by xanthine oxidase. The differences between the abilities of aldehyde oxidase and xanthine oxidase to oxidize various pteridine substrates are of particular interest in view of the similarity of the internal electron-transport systems of the two enzymes (46, 47), and such differences probably are best related to subtle differences in the environment of the catalytic site of these enzymes. Differences in the abilities of these enzymes to oxidize pteridine substrates are especially striking when comparisons are made of hydroxylated pteridines; xanthine oxidase appears to have a broader pattern of catalytic specificity with respect to oxidation of hydroxypteridines than does aldehyde oxidase. A

similar situation exists in the purine series where 8-hydroxypurine and 2,6-dihydroxypurine are oxidized readily by xanthine oxidase but not by aldehyde oxidase (5).

Cytochrome c reduction mediated by xanthine oxidase and, presumably, by aldehyde oxidase is associated with generation of superoxide free radicals by the flavoprotein enzyme system as a consequence of enzyme-catalyzed oxidation of a suitable substrate (48). Pteridine oxidations are conventional in the sense that the concomitant reduction of cytochrome c can be demonstrated to occur during enzymatic oxidation of 4-amino- and 2-aminopteridines, and these findings parallel those made in studies of analogous oxidations.

In summary, this study demonstrated that various pteridine derivatives are capable of being oxidized efficiently by mammalian liver xanthine oxidase and aldehyde oxidase; oxidations of pteridines mediated by liver xanthine oxidase follow reaction pathways essentially similar to those observed previously for the milk enzyme. Substantial differences exist between the susceptibilities of pteridines to oxidation by rat liver xanthine and rabbit liver aldehyde oxidase.

These studies have important implications for studies of pteridine metabolism *in vivo*; in fact, recent studies in this laboratory demonstrated extensive oxidation of pteridines *in vivo*, apparently mediated by both xanthine oxidase and aldehyde oxidase (49). It should be stressed that xanthine oxidase from various sources can be shown, under appropriate circumstances, to behave as an NAD^+-dependent dehydrogenase (50); the patterns of oxidation of pteridines by the "dehydrogenase" system may differ substantially from those observed for the "oxidase," and exploration of this possibility is planned.

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ACKNOWLEDGMENTS AND ADDRESSES

Received June 11, 1975, from the *Department of Pharmacology, College of Medicine, University of Vermont, Burlington, VT 05401*

Accepted for publication October 3, 1975.

Adapted in part from a dissertation submitted by C. N. Hodnett to the Graduate College, University of Vermont, in partial fulfillment of the Doctor of Philosophy degree requirements.

Supported in part by a U.S. Public Health Service Research Grant (CA 12876) from the National Cancer Institute, by a grant from the Vermont Division of the American Cancer Society, and by a predoctoral fellowship (NDEA Title IV) awarded to C. N. Hodnett.

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