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# OXIDATION OF PTERIDINE DERIVATIVES IN MICE

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**Abstract**—Studies were carried out of the metabolism of a variety of hydroxy-, mercapto- and aminopteridines following i.p. injection (150 mg/kg) into mice. Urinary oxidation products were isolated and identified by a combination of thin-layer chromatographic and spectrophotometric techniques. Pteridine and its 2- and 4-hydroxy derivatives as well as 2,4-dihydroxypteridine (lumazine) were converted extensively to 2,4,7-trihydroxypteridine. When mice were treated with the xanthine oxidase inhibitor allopurinol (20 mg/kg), formation of 2,4,7-trihydroxypteridine was sharply reduced (to about 25 per cent of its control value). Lumazine was detected in the urine of allopurinol-treated mice which received pteridine, 2-hydroxypteridine and 4-hydroxypteridine. Observations of hydroxypteridine oxidation in the absence and presence of allopurinol suggest that both xanthine oxidase and aldehyde oxidase can play a role in pteridine oxidation, *in vivo*. The administration of 2-aminopteridine to mice caused severe kidney toxicity, apparently attributable to the crystallization of highly insoluble oxidation products of this pteridine within the lumen of renal tubules. 4-Aminopteridine did not show such a nephrotoxic effect, but was extensively oxidized *in vivo* to 4-amino-2,7-dihydroxypteridine. Lumazine was found to be a major metabolic product when both 4-aminopteridine and allopurinol were administered. The formation of lumazine from 4-aminopteridine may involve the intermediate formation of 4-hydroxypteridine catalyzed by adenosine deaminase. 2-Mercapto and 4-mercaptopteridines were oxidized *in vivo* to compounds tentatively identified as the 4,7- and 2,7-dihydroxy derivatives, respectively. An additional compound, possibly resulting from cleavage of the pteridine ring, was detected in the urine of mice treated with 4-mercaptopteridine and allopurinol. A crude xanthine oxidase preparation from mouse liver was able to catalyze the oxidation of a number of pteridines previously found to be substrates for milk or rat liver xanthine oxidase. Catalytic constants ($K_m$ and $V_{max}$) using this mouse liver xanthine oxidase preparation were estimated for a number of pteridines. The patterns of substrate oxidation in such studies *in vitro* are consistent generally with those obtained following the i.p. administration of a number of pteridines to mice.

A number of investigations have been carried out to assess the susceptibility of pteridine derivatives to oxidation *in vitro* but little work has been done *in vivo*. We have been interested in the oxidation of various pteridines by the metalloflavoenzymes, xanthine oxidase and aldehyde oxidase. Early work in this area by Bergmann and Kwietny [1, 2] showed that hydroxysubstituted pteridines are substrates for milk xanthine oxidase. These observations were extended in this laboratory to include aminopteridines [3], and further extended when Hodnett, also in this laboratory, demonstrated that various amino- and hydroxypteridines are substrates for rat liver xanthine oxidase, as well as for rabbit liver aldehyde oxidase [4]. The susceptibility to oxidation (i.e. hydroxylation of the ring system) of certain pteridines by aldehyde oxidase has also been investigated by Krenitsky and his colleagues [5].

Previous studies have been performed on the metabolism *in vivo* of triamterene [6], methotrexate [7], 2,4-diamino-6,7-dimethylpteridine [8], and various 6-alkyl substituted pteridines [9, 10], but a systematic study using a variety of substituted pteridines has not been performed. We therefore wished to observe whether the oxidation of pteridines, *in vivo*, follows pathways analogous to those previously observed using isolated enzymes. A recent report [11] that isoxanthopterin (2-amino-4,7-dihydroxypteridine) may possess antitumor activity, suggested that our studies of the metabolic alterations of pteridine derivatives might serve to identify precursor compounds (e.g. 2-aminopteridine) which may be transformed into metabolically active agents (e.g. isoxanthopterin).

## MATERIALS AND METHODS

*Materials.* Pteridines used in this study were synthesized by established procedures described in references contained in the appropriate tables. Both catalase (Worthington Biochemical Corp., Freehold, NJ) and milk xanthine oxidase (Nutritional Biochemicals, Cleveland, OH) were obtained commercially and used without further purification. Aldehyde oxidase was prepared from frozen rabbit livers (Pel-Freez, Inc., Rogers, AR) according to the procedure of Wolpert, *et al.* [12]. Xanthine oxidase from mouse tissues was prepared by the following procedure. Animals were killed by a blow to the head, and tissues were rapidly excised and placed in ice-cold Tris buffer (0.05 M; pH 7.8, containing 0.005% EDTA), then blotted dry, weighed, and homogenized in 5 volumes of the same buffer, using a Potter–Elvehjem hand homogenizer with a teflon pestle. The crude homogenate was centrifuged at 2000 rpm for 10 min, and the supernatant solution from this centrifugation was then centrifuged at 75,000 rpm for 40 min in a Beckman Model L ultracentrifuge at 4°. The supernatant solution from this centrifugation was dialysed 12 hr against 100

volumes of Tris/EDTA buffer in the cold. Ammonium sulfate fractionation (30% and 55% saturation) was performed on the dialysate. The protein which precipitated from the 55% ammonium sulfate fraction was dissolved in Tris buffer and used as the xanthine oxidase source. Xanthine oxidase activity was followed throughout this purification procedure by using 4-hydroxypteridine as a substrate ($1 \times 10^{-4}$ M) and measuring the absorbance change at 328 nm. Aldehyde oxidase activity (as a contaminant in the xanthine oxidase preparations) was monitored at 300 nm using *N*-methylnicotinamide ($3 \times 10^{-3}$ M) as a substrate. Aldehyde oxidase activity in these preparations could be completely inhibited by the addition of menadione ($1 \times 10^{-5}$ M) to the system. The amount of aldehyde oxidase present in the preparation varied from nil (none detectable) to about 5 per cent of "pteridine oxidizing activity"; for example, in the latter case only 5 per cent of the oxidation of 4-hydroxypteridine could be attributed to aldehyde oxidase.

*Methods.* The following procedure was used for assaying the susceptibility to oxidation of pteridine derivatives by either milk or mouse liver xanthine oxidase. Substrate solution (0.1 ml; prepared by dissolving 1.0 mg of the desired compound in 10.0 ml $H_2O$) was added to 0.9 ml of phosphate buffer (0.1 M, pH 7.0) in a 1 ml quartz cuvette. Catalase (151 units/ml) was added to minimize any enzyme inactivation due to $H_2O_2$ liberation during the course of the enzymatic reaction. Following addition of catalase, 10 $\mu$l of an ethanolic menadione solution (final concentration $1 \times 10^{-5}$ M), was added to inhibit any aldehyde oxidase activity which might have been present in the xanthine oxidase preparation. Finally a quantity of xanthine oxidase (usually 20–50 $\mu$l of a stock solution), sufficient to give an absorbance change of 0.5/10 min, at 290 nm, evaluated using hypoxanthine ($1 \times 10^{-4}$ M) as substrate, was added. A reference cuvette, containing all of the above components except substrate, was used as a blank. All spectral analyses of enzymatic reactions were performed at 37° using a Perkin–Elmer Model 202 double-beam spectrophotometer. A reaction was considered complete when no detectable absorbance changes could be observed on further incubation of the reaction mixture. For inhibition studies, the xanthine oxidase inhibitor allopurinol was employed at a concentration ($10^{-4}$ M) which has been shown to effectively inhibit pteridine oxidation, *in vitro* [3].

The initial rates of oxidation of a variety of pteridines, mediated by mouse liver xanthine oxidase, were estimated using a Beckman Model 25 double-beam kinetic spectrophotometric system. For those experiments the enzymatic system contained substrate (10 $\mu$g/ml), catalase (151 units/ml) and menadione ($1 \times 10^{-5}$ M) in phosphate buffer (pH 7.0). Xanthine oxidase was added to start the reaction and the absorbance increase at a fixed wavelength was followed for 20 min, during which time the initial rate was linear for all substrates. The final rates were calculated as $\mu$moles oxidized/l/hr. Kinetic constants ($K_m$ and $V_{max}$) for some pteridines were estimated graphically according to the method of Lineweaver and Burk [13]. For these estimations substrate concentrations of 2, 4, 6 and 8 $\mu$g/ml were employed. The $K_m$ value ($4 \times 10^{-6}$ M) and relative $V_{max}$ for 2,4-dihydroxypteridine, which is not a substrate for aldehyde oxidase, were not affected by the presence of menadione in the mouse liver system.

For *in vivo* experiments female CD-1 mice (Canadian Breeding Laboratories) were divided into 3 groups of 5 each. One group received an i.p. injection of 0.5 ml of a pteridine suspension (in 0.9% sodium chloride solution; total dose 150 mg/kg), while another group received an injection of a pteridine, as above, but followed immediately by 0.25 ml of an allopurinol solution (in 0.9% sodium chloride; total dose 20 mg/kg). This dose of allopurinol has been shown to effectively inhibit the oxidation of 6-mercaptopurine, a xanthine oxidase substrate, *in vivo* [14]. A third group of animals received i.p. injections of saline solutions and served as the control group. In two instances (i.e., those of the highly insoluble pteridines, 2-amino-4-hydroxypteridine and 2-amino-4,7-dihydroxypteridine) it was necessary to administer the compounds orally, because i.p. injection of a suspension of either of these pteridines in saline was followed by a deposition of the pteridine in the peritoneal cavity. Following injections, mice were placed in metabolism cages and the urine was collected at various intervals thereafter, up to 24 hr. The collected urine was centrifuged to remove particulate matter and aliquots of the supernatant were chromatographed on thin-layer chromatography plates (Analtech, Newark, DE). The fluorescent spots on the TLC plates were visualized under u.v. light, scraped from the plates and eluted using a small volume of Tris buffer. The eluted material was then subjected to u.v.-visible spectral analysis, using as a reference the elution from a comparable area of a chromatogram of urine from control mice.

Identification of a tentative oxidation product in the urine of mice following administration of a particular pteridine was made by comparing the chromatographic and spectral characteristics (spectra taken at pH 7.8, and 13) of the suspected compound with those of an authentic sample. The concentration of a pteridine in the urine was estimated by comparing the extinction of a solution of the material following its elution from the t.l.c. plate with that of a solution of authentic compound of known concentration. In those cases where pure reference compounds were not available, such compounds were prepared enzymatically by incubating a pteridine with milk xanthine oxidase or rabbit liver aldehyde oxidase. The chromatographic and spectral properties of the enzymatically prepared compound were then used to identify and quantitate urinary pteridines. In some cases a further characterization of substances eluted from t.l.c. plates was carried out by adding either milk xanthine oxidase or rabbit liver aldehyde oxidase to the solution of the material eluted from the t.l.c. plate and determining the nature of the products obtained on enzymatic oxidation.

In some instances, after the administration of aminopteridines, mice were sacrificed and pteridines were extracted from their kidneys. Kidneys were homogenized with 5 volumes of cold Tris/EDTA buffer using a hand-held homogenizer. Centrifugation at 2000 rpm for 10 min yielded a supernatant from which protein was precipitated using a combination of 0.3 N $Ba(OH)_2$ and 5% $Al_2(SO_4)_3$ according to the

procedure of Gyure [15]. The protein-free extracts so obtained were subjected to chromatographic and spectral analysis. Microscope slides (stained with hematoxylin and eosin) were also prepared from sections of kidneys of some mice, which had received certain aminopteridines.

## RESULTS

The results of chromatographic and spectral analyses of the urine of mice which received pteridine and various hydroxypteridines are given in Table 1. They indicate that pteridine when administered to mice, is converted exclusively to 2,4,7-trihydroxypteridine. The administration of allopurinol, along with pteridine, decreased the amount of this metabolite in the urine to about 1/3 of its control value (no allopurinol administration). The major product in the urine of allopurinol-treated mice was 2,4-dihydroxypteridine (lumazine). A small amount of a third product, identified as 4-hydroxypteridine, was also found in the urine of these allopurinol-treated animals. The identification of this product was based on its $R_f$ value, spectral properties, and the fact that it is converted to 2,4,7-trihydroxypteridine when incubated with milk xanthine oxidase (a reaction characterized by a great increase in extinction at 330 nm [2]). We noted, during the experiments with pteridine, that this compound is able to induce convulsions in mice; similar convulsant activity has previously been observed when high doses of 6-chloropurine are administered to experimental animals [16].

Following administration of 4-hydroxypteridine to mice, a small amount of unchanged material was detected in the urine, as well as a much greater amount of 2,4,7-trihydroxypteridine. The unmetabolized 4-hydroxypteridine, after elution from the t.l.c. plate, was converted to lumazine by incubation with rabbit liver aldehyde oxidase, an observation which confirmed its identity. When allopurinol was administered together with 4-hydroxypteridine to mice, 3 compounds were detected in the urine. The major product in this case was lumazine; 4-hydroxypteridine and 2,4,7-trihydroxypteridine were present at lower levels.

When lumazine was administered to mice a large amount of 2,4,7-trihydroxypteridine was excreted, along with a lesser amount of unchanged lumazine. Allopurinol administration resulted in a considerable alteration of the proportion of these compounds present in urine; for example, the urinary level of 2,4,7-trihydroxypteridine fell by about 75 per cent, while the amount of lumazine increased 20-fold.

Two principal products were found in the urine following administration of 2-hydroxypteridine to mice, a relatively large amount of 2,4,7-trihydroxypteridine and a smaller amount of a second compound. The nature of the latter substance is unclear, but it appears to be related to 3,4-dihydro-2-hydroxypteridine on the basis of the similarity of its spectral properties to those of this compound [19]. A third fluorescent product was detected in urine of 2-hydroxypteridine-treated mice, but at a concentration too low to be amenable to ultraviolet spectral analysis. Four compounds were present in the urine of mice treated with 2-hydroxypteridine and allopurinol. 2,4,7-Trihydroxypteridine was present at about 1/3 the concentration of that found in urine of animals not treated with allopurinol. A small amount of unchanged 2-hydroxypteridine, as well as lumazine

Table 1. Analysis of urine from mice which received pteridine and various hydroxy-substituted pteridines

| Compounds administered | $R_f$ of spots located | Spectral maxima (nm) pH 7.8 | pH 13.0 | Proposed identity | Amount recovered (mg) | Percent of administered dose |
|---|---|---|---|---|---|---|
| Pteridine [17] | 0.31 | — | — | — | — | —† |
| | 0.06 | 328, 340 (s)* | 273, 335 | 2,4,7-OH Pt | 8.1 | 54 |
| Pteridine and allopurinol | 0.40 | 270, 330 | 255, 370 | 2,4-OH Pt | 5.8 | 39‡ |
| | 0.24 | 330 | — | 4-OH Pt | 0.1 | —§ |
| | 0.05 | 328, 340 (s) | 276, 334 | 2,4,7-OH Pt | 2.9 | 19 |
| 4-OH Pteridine [18] | 0.26 | 245, 326 | 335 | 4-OH Pt | 0.6 | 4§ |
| | 0.06 | 328, 340 (s) | 275, 336 | 2,4,7-OH Pt | 8.7 | 58 |
| 4-OH Pteridine and allopurinol | 0.40 | 270, 330 | 255, 370 | 2,4-OH Pt | 5.3 | 35‡ |
| | 0.24 | 328 | 335 | 4-OH Pt | 2.1 | 14§ |
| | 0.06 | 328, 340 (s) | 275, 336 | 2,4,7-OH Pt | 2.0 | 13 |
| 2,4-OH Pteridine‖ | 0.39 | 270, 330 | 255, 370 | 2,4-OH Pt | 0.3 | 2‡ |
| | 0.05 | 328, 340 (s) | 276, 336 | 2,4,7-OH Pt | 6.3 | 42 |
| 2,4-OH Pteridine and allopurinol | 0.40 | 270, 330 | 255, 370 | 2,4-OH Pt | 6.7 | 45‡ |
| | 0.06 | 328, 340 (s) | 275, 337 | 2,4,7-OH Pt | 1.6 | 11 |
| 2-OH Pteridine [17] | 0.28 | 246, 318 | 342 | 2-OH,3,4-dihydro Pt | 1.5 | 10¶ |
| | 0.07 | 328, 339 (s) | 276, 335 | 2,4,7-OH Pt | 8.6 | 57 |
| 2-OH Pteridine and allopurinol | 0.55 | 308 | 314 | 2-OH Pt | 1.0 | 7‡ |
| | 0.41 | 270, 329 | 370 | 2,4-OH Pt | 1.8 | 12‡ |
| | 0.26 | 246, 318 | 278, 345 | 2-OH-3,4-dihydro Pt | 1.6 | 11¶ |
| | 0.06 | 328, 340 (s) | 275, 335 | 2,4,7-OH Pt | 2.5 | 17 |

* (s) Denotes a shoulder in the absorption spectrum.

† Insufficient amount of material present in urine to permit analysis.

‡ Material eluted from the t.l.c. plate forms 2,4,7-trihydroxypteridine when incubated with milk xanthine oxidase. The material is not a substrate for rabbit liver aldehyde oxidase.

§ Material eluted from the t.l.c. plate is a substrate for both rabbit liver aldehyde oxidase (optical density change suggestive of formation of 2,4-dihydroxypteridine) and milk xanthine oxidase (optical density change suggestive of formation of 2,4,7-trihydroxypteridine formation).

¶ Material not a substrate for milk xanthine oxidase.

‖ Obtained commercially (Aldrich Chemical Company).

*Abbreviations*: —OH; hydroxy: Pt; pteridine.

*Chromatographic System for hydroxypteridines*: Avicel (Analtech) thin-layer plates Butanol/DMF/$H_2O$ (65:25:10) as solvent system.

Table 2. Analysis of urine from mice which received 2-aminopteridine or its hydroxylated derivatives

| Compounds administered | $R_f$ of spots located | Spectral maxima (nm) pH 7.8 | pH 13.0 | Tentative identity | Amount recovered (mg) | Percent of administered dose Urine | Kidneys | Feces |
|---|---|---|---|---|---|---|---|---|
| 2-$NH_2$ Pteridine [17] | 0.33 | 268, 344 | — | 2-$NH_2$-4-OH or 2-$NH_2$-7-OH pteridine | 0.1 | — | — | — |
| | 0.06 | 282, 335 | 342 | 2-$NH_2$-4,7-OH pteridine | 5.4 | 17 | 19 | |
| 2-$NH_2$ Pteridine and allopurinol | 0.35 | 270, 345 | — | 2-$NH_2$-4-OH or 2-$NH_2$-7-OH pteridine | 1.8* | 2 | 10 | |
| | 0.06 | 280, 335 | 254, 342 | 2-$NH_2$-4,7-OH pteridine | 3.5 | 13 | 11 | |
| 2-$NH_2$-4-OH†§ Pteridine | 0.34 | 268, 346 | 360 | 2-$NH_2$-4-OH pteridine | 6.5 | 3 | 4 | 36 |
| | 0.05 | 335 | 342 | 2-$NH_2$-4,7-OH pteridine | 2.8 | 9 | 9 | — |
| 2-$NH_2$-4-OH Pteridine and allopurinol | 0.36 | 267,345 | 360 | 2-$NH_2$-4-OH pteridine | 8.2 | 9 | 7 | 39 |
| | 0.05 | 335 | 342 | 2-$NH_2$-4,7-OH pteridine | 2.1 | 5 | 9 | — |
| 2-$NH_2$-4,7-OH Pteridine‡§ | 0.08 | 335 | 342 | 2-$NH_2$-4,7-OH pteridine | 6.0 | 13 | 8 | 46 |

* This material could not be positively identified; eluted material was converted to 2-$NH_2$-4,7-OH pteridine upon incubation with milk xanthine oxidase; the amount recovered was estimated by comparing the extinction of the unknown with that of a solution of known concentration of 2-$NH_2$-4-OH pteridine.

† 15.0 mg of this pteridine were administered as an aqueous suspension orally to a group of 5 mice.

‡ 9 mg of this pteridine were administered as an aqueous suspension orally to a group of 5 mice (Aldrich Chem. Co.).

§ Obtained commercially.

*Chromatographic System for Aminopteridines*: Cellulose thin-layer plates (Analtech) EtOH/$NH_3$/$H_2O$ (80:10:10) as solvent system.

(identified by its $R_f$ value, spectral properties, ability to form 2,4,7-trihydroxypteridine when incubated with milk xanthine oxidase, and inertness to rabbit liver aldehyde oxidase) were also detected in the urine of these animals. In addition to these compounds, the two unidentified compounds, observed in the urine of mice receiving only 2-hydroxypteridine were also found in the allopurinol-treated mice.

The results of the chromatographic and spectral analyses of urine, as well as kidney and fecal extracts, from mice which received 2-aminopteridine and its hydroxylated derivatives are shown in Table 2. Following the administration of 2-aminopteridine (150 mg/kg) to mice all animals died within 12–24 hr. A striking reduction in urine output was observed in these animals prior to death, thus suggesting that this pteridine may be affecting the kidneys. The urine output was not reduced as sharply in those animals which also were treated with allopurinol, nor did those animals die. As shown in Table 2, the kidneys of both groups of animals contained hydroxylated derivatives of 2-aminopteridine. In 2-aminopteridine-treated mice the only compound detectable in the kidneys was 2-amino-4,7-dihydroxypteridine (isoxanthanopterin), while in allopurinol-treated mice both this compound and a monohydroxy-2-aminopteridine were found (due to similarities in $R_f$'s of 4-hydroxy- and 7-hydroxy-2-aminopteridine in the chromatographic system used, these isomers could not be clearly separated in the kidney extracts). Histologic examination of kidney sections from mice which received 2-aminopteridine revealed widespread degeneration and necrosis of cortical tubules. Cellular debris and other material (apparently hyaline casts) was found in the lumen of cortical and medullary tubules. The presence of a foreign granular material was noted throughout the sections. There appeared to be no damage to the glomeruli or renal vasculature. By contrast, no significant pathology was evident upon examination of kidney sections from mice which had been treated with both 2-aminopteridine and allopurinol. Foreign granular material was present, however, in the collecting ducts and/or the ascending limb of Henle's loop, primarily in corticomedullary tubules, of mice treated with 2-aminopteridine and allopurinol. The foreign material present in the kidneys of 2-aminopteridine treated mice appears to be the isoxanthopterin which was readily detected in kidney extracts. As both isoxanthopterin and either 2-amino-4- or 7-hydroxypteridine were detected in the kidneys of allopurinol-treated mice, the foreign material present in kidney sections from these animals is presumably a mixture of these compounds. As noted in Table 2, much less of the administered dose of either 2-amino-4-hydroxypteridine or isoxanthopterin could be detected in the kidneys than could be found in these organs after a dose of 2-aminopteridine, probably due to lack of absorption of these poorly soluble pteridines after oral administration.

Table 3 presents the results of chromatographic and spectral analyses of urine from mice which received either 4-aminopteridine or 2,4-diaminopteridine, with or without allopurinol. It should be noted that, in neither case, could pteridines be detected in kidney extracts from mice which received these pteridines. Following the administration of 4-aminopteridine, a small amount of the injected material, as well as a large amount of 4-amino-2,7-dihydroxypteridine, could be detected in urine. When allopurinol was also administered, 4-aminopteridine, 4-amino-2,7-dihyd-

Table 3. Analysis of urine from mice which received either 4-aminopteridine or 2,4-diaminopteridine

| Compounds administered | $R_f$ of spots located | Spectral maxima (nm) pH 7.8 | pH 13.0 | Tentative identity | Amount recovered (mg) | Percent of administered dose |
|---|---|---|---|---|---|---|
| 4-$NH_2$ Pteridine | 0.44 | 274 (s),* 336 | 244, 335 | 4-$NH_2$ Pt | 1.2 | 8† |
| [17] | 0.09 | 335, 348 (s) | 335, 346 (s) | 4-$NH_2$-2,7-OH pteridine | 6.4 | 43 |
| 4-$NH_2$ Pteridine | 0.41 | 274 (s), 337 | 244, 335 | 4-$NH_2$ Pt | 2.3 | 15† |
| and allopurinol | 0.30 | 270, 330 | 255, 370 | 2,4-OH Pt | 1.7 | 11‡ |
| | 0.17 | 333, 344 (s) | 322, 330, 344 (s) | 4-$NH_2$-7-OH pteridine | 1.6 | 11† |
| | 0.07 | 336, 347 (s) | 335, 346 (s) | 4-$NH_2$-2,7-OH pteridine | 2.0 | 13 |
| 2,4-$NH_2$ Pteridine | 0.27 | 257, 365 | — | 2,4-$NH_2$ Pt | 3.5 | 23§ |
| [20] | 0.12 | 353 | — | 2,4-$NH_2$-7-OH pteridine | 0.8 | 5‖ |
| 2,4-$NH_2$ Pteridine | 0.25 | 256, 365 | — | 2,4-$NH_2$ Pt | 6.2 | 41§ |
| and allopurinol | 0.11 | 353 | — | 2,4-$NH_2$-7-OH pteridine | 1.0 | 7‖ |

* (s) Denotes a shoulder in the absorption spectrum.
† Substrate for milk xanthine oxidase, forming 4-amino-2,7-dihydroxypteridine upon incubation.
‡ Substrate for milk xanthine oxidase, forming 2,4,7-trihydroxypteridine upon incubation.
§ Substrate for rabbit liver aldehyde oxidase, forming 2,4-diamino-7-hydroxypteridine upon incubation.
‖ Not a substrate for rabbit liver aldehyde oxidase.
*Chromatographic System for Aminopteridines*: Cellulose thin-layer plates (Analtech) EtOH/$NH_3$/$H_2O$ (80:10:10) as solvent system.

roxypteridine, and an intermediate oxidation product, 4-amino-7-hydroxypteridine, could all be detected in urine. Lumazine was also demonstrated to be present in the urine of allopurinol-treated mice. Following administration of 2,4-diaminopteridine, with or without allopurinol, only 2 substances were detected in the urine; these substances were identified as 2,4-diamino-7-hydroxypteridine and 2,4-diaminopteridine. The amount of 2,4-diamino-7-hydroxypteridine found was low relative to that of unoxidized 2,4-diaminopteridine in both cases. Although we noted no gross alteration of kidney function in mice receiving a single injection of 2,4-diaminopteridine, it should be stressed that this pteridine administered to rats daily for a period of 10 days did produce pathological changes in kidney, associated with deposition of an unknown fluorescent substance in kidney tissue [21].

Table 4 summarizes the spectral changes seen upon incubation of three mercaptopteridines with either milk xanthine oxidase or rabbit liver aldehyde oxidase. No spectral changes were seen when any of these compounds were incubated with aldehyde oxidase, but all three compounds were altered when incubated with xanthine oxidase. Such xanthine oxidase-induced spectral changes were inhibited (>95 per cent) by allopurinol ($10^{-4}$ M).

The analysis of urine from mice which received each of these mercaptopteridines is shown in Table 5. Of the three compounds 2-mercaptopteridine appeared to be oxidized least rapidly, *in vivo*. When 4-mercaptopteridine and allopurinol were administered to mice, a third compound, which appeared to represent the major product, was detected in the urine. The nature of this material was not established, although it may represent a compound formed by degradation of the pteridine ring system, by a process similar to that observed in chemical systems by Taylor *et al.* [24].

Table 6 summarizes the initial rates of oxidation of a number of pteridines which were found to be substrates for mouse liver xanthine oxidase. All of these pteridines were also shown to be capable of being oxidized *in vivo*. Table 7 summarizes the kinetic constants ($K_m$ and $V_{max}$) estimated for a number of pteridines.

## DISCUSSION

Pteridine, 2- and 4-hydroxypteridines and 2,4-dihydroxypteridine (lumazine) are each converted to 2,4,7-trihydroxypteridine by milk xanthine oxidase [2] and xanthine oxidase isolated from rat liver [4]. Rabbit liver oxidizes pteridine to lumazine (apparently via 4-hydroxypteridine as an intermediate), 4-hydroxypteridine to lumazine, and both lumazine

Table 4. The action of milk xanthine oxidase and rabbit liver aldehyde oxidase on mercaptopteridines

| Compound | Conc. ($\times 10^{-5}$ M) | Enzyme | u.v. Maxima Start | After 24 hr |
|---|---|---|---|---|
| 2-Mercaptopteridine | 6.1 | X.O. | 272, 325 | 260, 338, 353 |
| | | A.O. | 271, 314 | 271, 313 |
| 2-Mercapto-4-hydroxypteridine | 5.6 | X.O. | 306, 360 | 260, 338, 353 |
| | | A.O. | 306, 363 | 305, 362 |
| 4-Mercaptopteridine | 6.1 | X.O. | 262, 400 | 280, 370, 382 |
| | | A.O. | 272, 411 | 273, 410 |

Xanthine oxidase (X.O.) reactions run in phosphate buffer, pH 7.0 at 37°.
Aldehyde oxidase (A.O.) reactions run in Tris/EDTA buffer, pH 7.8 at 37°, in the presence of $1 \times 10^{-4}$ M allopurinol.

Table 5. Analysis of urine from mice which received either 2-, or 4-mercaptopteridine or 2-mercapto-4-hydroxypteridine

| Compounds administered | $R_f$ of spots located | Spectral maxima (nm) pH 7.8 | pH 13.0 | Tentative identity | Amount recovered (mg) | Percent of administered dose |
|---|---|---|---|---|---|---|
| 2-SH Pteridine [22] | 0.77 | 274, 322 | 291, 344 | 2-SH Pt | 3.2 | 21† |
| | 0.54 | 340, 353 | 306, 347 | 2-SH-4,7-OH pteridine | 5.1 | 34 |
| 2-SH Pteridine and allopurinol | 0.75 | 276, 321 | 290, 344 | 2-SH Pt | 6.1 | 41† |
| | 0.66 | 307, 360 | 286, 376 | 2-SH-4-OH Pt | 0.5 | 3† |
| | 0.52 | 340, 353 | 305, 348 | 2-SH-4,7-OH pteridine | 1.8 | 12 |
| 2-SH-4-OH* | 0.68 | 307, 360 | 286, 376 | 2-SH-4-OH Pt | 0.9 | 6† |
| Pteridine | 0.53 | 340, 353 | 305, 347 | 2-SH-4,7-OH Pt | 5.1 | 34 |
| 2-SH-4-OH | 0.67 | 307, 360 | 287, 376 | 2-SH-4-OH Pt | 8.2 | 55† |
| Pteridine and allopurinol | 0.54 | 340, 353 | 305, 347 | 2-SH-4,7-OH Pt | 1.5 | 10 |
| 4-SH Pteridine [23] | 0.56 | 272, 406 | 265, 408 | 4-SH Pt | 1.0 | 7‡ |
| | 0.09 | 283, 370, 382 | 283, 374 | 4-SH,2,7-OH Pt | 8.7 | 58 |
| 4-SH Pteridine and allopurinol | 0.54 | 408 | 264, 410 | 4-SH Pt | 2.1 | 14‡ |
| | 0.08 | 370, 382 | 283, 375 | 4-SH-2,7-OH Pt | 2.0 | 13 |

* Obtained commercially (Aldrich Chem. Co.).

† Substrate for milk xanthine oxidase, forming 2-mercapto-4,7-dihydroxypteridine upon incubation.

‡ Substrate for milk xanthine oxidase, forming 4-mercapto-2,7-dihydroxypteridine upon incubation.

*Chromatographic System for mercaptoperidines*: silica gel G-plates with fluorescent indicator (Analtech) Butanol/HOAc/$H_2O$ (66:10:24) as solvent system.

Table 6. Rates of oxidation of hypoxanthine and various pteridines with a xanthine oxidase preparation from mouse liver*†

| Compound | Analytical wavelength | Oxidation rate (μmoles oxidized/l/hr) | % Rate relative to hypoxanthine |
|---|---|---|---|
| Hypoxanthine (pteridines) | 290 ($12 \times 10^3$)‡ | 112.8 | — |
| Unsubstituted | 328 ($12.6 \times 10^3$) | 32.9 | 29 |
| 2-hydroxy- | 328 ($11.8 \times 10^3$) | 16.0 | 14 |
| 4-hydroxy- | 328 ($8.3 \times 10^3$) | 50.9 | 45 |
| 2,4-dihydroxy- | 328 ($5.3 \times 10^3$) | 108.8 | 96 |
| 2-amino- | 335 ($8.5 \times 10^3$) | 40.1 | 36 |
| 2-amino-4-hydroxy- | 335 ($7.6 \times 10^3$) | 115.7 | 103 |
| 4-amino- | 335 ($9.3 \times 10^3$) | 26.9 | 24 |
| 2-mercapto- | 338 ($11 \times 10^3$) | 16.4 | 15 |
| 2-mercapto-4-hydroxy- | 338 ($9.1 \times 10^3$) | 46.1 | 41 |
| 4-mercapto- | 370 ($13 \times 10^3$) | 33.1 | 29 |

* All reactions carried out in the presence of menadione ($1 \times 10^{-5}$ M).

† All substrates evaluated at a concentration of 10 μg/ml.

‡ Numbers in parentheses represent the change in extinction coefficient which characterizes the enzymatic reaction.

Table 7. Apparent kinetic constants obtained using mouse liver xanthine oxidase

| Compound | $K_m$ ($\times 10^{-5}$ M) | $V_{max}$ (μmoles oxidized/l/hr) |
|---|---|---|
| 4-OH Pteridine | 7.2 | 132 |
| 4-SH Pteridine | 2.8 | 108 |
| 2-$NH_2$ Pteridine | 8.3 | 77 |
| 2-$NH_2$-4-OH-Pteridine | 1.4 | 166 |
| 4-$NH_2$ Pteridine | 17.5 | 51 |

and 2-hydroxypteridine are refractory to oxidation by this enzyme [4, 5].

Pteridine and its hydroxylated derivatives are extensively oxidized, *in vivo*. The finding of 2,4,7-trihydroxypteridine as the major product in the urine of mice which received these compounds, as well as the decrease in the urinary levels of this pteridine when allopurinol was also administered, support a role for xanthine oxidase in pteridine oxidation, *in vivo*. Although mouse liver xanthine oxidase oxidizes a variety of pteridines, *in vitro* (Table 6), the possibility that extra-hepatic xanthine oxidase also mediates pteridine oxidation cannot be ruled out by this work. The finding of lumazine in the urine of mice which received either pteridine and allopurinol or 4-hydroxypteridine and allopurinol indicates that aldehyde oxidase may also play a role in oxidation of pteridines *in vivo*. The detection of a small amount of 4-hydroxypteridine in the urine of mice treated with pteridine and allopurinol, is consistent with the observation, that this compound is an intermediate in the oxidation of pteridine by aldehyde oxidase [4]. The absence of pteridine in the urine of mice treated with pteridine and allopurinol suggests that pteridine is oxidized rapidly by aldehyde oxidase, *in vivo*. This finding is consistent with the observation made by Krenitsky *et al.* [5], that pteridine is oxidized more rapidly than 4-hydroxypteridine by rabbit liver aldehyde oxidase, *in vitro*.

The presence of lumazine in the urine of mice treated with 2-hydroxypteridine and allopurinol may be due to oxidation of 2-hydroxypteridine mediated by aldehyde oxidase; it should be emphasized, in this regard, however, that 2-hydroxypteridine is not an effective substrate for rabbit liver aldehyde oxidase.

The observation of lumazine formation from 2-hydroxypteridine *in vivo* might reflect a difference in substrate specificity between mouse and rabbit liver aldehyde oxidase; such a difference in the abilities of these mammalian aldehyde oxidases to oxidize another pteridine substrate, methotrexate, has previously been established [7, 25].

Results generally consistent with previous observations *in vitro* [3, 4] were obtained in the present study following the administration of 2-aminopteridine to animals (Table 2). The amount of recovered isoxanthopterin from kidney extracts and urine was 1/3 lower in mice which received 2-aminopteridine and allopurinol, compared to the amount observed in animals which received 2-aminopteridine only. This finding indicates that xanthine oxidase can mediate the oxidation of 2-aminopteridine, *in vivo*. The potential for renal toxicity associated with 2-aminopteridine administration, alluded to earlier, should again be stressed, especially in light of recent interest in isoxanthopterin as a potential antineoplastic agent [11]. The recovery of isoxanthopterin from kidneys of mice treated with 2-aminopteridine suggests that the deposition of this highly insoluble pteridine within the kidney is associated with the renal toxicity. The lack of kidney pathology observed in kidneys from mice treated with 2-aminopteridine and allopurinol is presumably associated with the lesser amount of isoxanthopterin present in the kidney under these conditions. It should be pointed out that other pteridine derivatives such as folic acid and xanthopterin (2-amino-4,6-dihydroxypteridine) are known to produce physical damage to the kidney and this damage results in hypertrophic changes associated with increased DNA synthesis [26].

4-Aminopteridine is converted to 4-amino-2,7-dihydroxypteridine by milk xanthine oxidase [3] and rat liver xanthine oxidase. 4-Aminopteridine is converted to 4-amino-7-hydroxypteridine by rabbit liver aldehyde oxidase [4]. Neither 4-amino-7-hydroxypteridine nor its 2-hydroxy isomer are substrates for aldehyde oxidase, but both are oxidized to 4-amino-2,7-dihydroxypteridine by milk and rat liver xanthine oxidase [3, 4]. The fairly extensive conversion of 4-aminopteridine to 4-amino-2,7-dihydroxypteridine in mice (Table 3), along with the decreased formation of the latter compound in allopurinol-treated animals, suggest a role for xanthine oxidase in the oxidation of 4-aminopteridine, *in vivo*. The administration of allopurinol results in the accumulation of the compound formed by the action of aldehyde oxidase on 4-aminopteridine (i.e., 4-amino-7-hydroxypteridine) in the urine. The lumazine detected in the urine of mice treated with 4-aminopteridine and allopurinol probably arises through a deamination reaction. 4-Aminopteridine has been shown to be a substrate for adenosine deaminase (from *Aspergillus oryzae* and calf intestine), forming 4-hydroxypteridine [27]. The 4-hydroxypteridine so formed can be converted to lumazine by aldehyde oxidase, *in vivo*. With the exception of this proposed adenosine deaminase-catalyzed reaction, 4-aminopteridine appears to be oxidized *in vivo* via reaction pathways similar to those observed using isolated enzymes, *in vitro*.

2,4-Diaminopteridine is not a substrate for milk xanthine oxidase [28] while this compound, as well as its 6-methyl and 6-hydroxymethyl derivatives, is oxidized to the corresponding 7-hydroxy derivatives by rabbit liver aldehyde oxidase. That oxidation of a 2,4-diaminopteridine can be mediated by aldehyde oxidase was established first by Johns and his colleagues [7], who demonstrated that this enzyme oxidizes methotrexate to 7-hydroxy methotrexate [29].

In the present study, both 2,4-diaminopteridine and its 7-hydroxy derivative were found in the urine from animals which had received the former pteridine (Table 3). That allopurinol administration did not alter the relative proportions of these compounds is consistent with the observation that 2,4-diaminopteridine is not a xanthine oxidase substrate, *in vitro*. That the amount of 2,4-diamino-7-hydroxypteridine found in the urine is small relative to that of 2,4-diaminopteridine, is not surprising in light of previous studies [7, 25] which demonstrated a low rate of hydroxylation of methotrexate in the mouse relative to the rabbit. The behavior of 2,4-diaminopteridine, *in vivo*, therefore, closely resembles the oxidation process observed *in vitro*. It should be noted in this connection that 7-hydroxylation of the diaminopteridine derivative methotrexate appears to be of clinical significance in patients who are undergoing "high-dose" methotrexate therapy [30].

Since mercaptopteridines have not been previously examined as possible substrates for xanthine or aldehyde oxidases, we decided to investigate the actions of these enzymes on selected mercaptopteridines *in vitro* (Table 4) and the pattern of oxidation of these mercaptopteridines *in vivo* (Table 5). 2-Mercaptopteridine appeared to be a substrate for both mouse liver and milk xanthine oxidase, but not for rabbit liver aldehyde oxidase. The product of the reaction of 2-mercaptopteridine and xanthine oxidase is identical both spectrophotometrically and chromatographically with that formed from 2-mercapto-4-hydroxypteridine and xanthine oxidase and it is inferred, therefore, that the product is 2-mercapto-4,7-dihydroxypteridine. The rather large amount of unchanged 2-mercaptopteridine detected in the urine of mice which received this pteridine parallels the observation that this pteridine reacts at a fairly slow rate with mouse liver xanthine oxidase, *in vitro* (as judged by initial rate studies; Table 6). The small amount of 2-mercapto-4-hydroxypteridine found in the urine of mice treated with 2-mercaptopteridine and allopurinol may arise from the action of mouse aldehyde oxidase on 2-mercaptopteridine. Since rabbit liver aldehyde oxidase was used for *in vitro* studies, the occurrence of an "aldehyde oxidase product" in mice could represent a species difference in this enzyme in rabbits and mice. We conclude that 2-mercaptopteridine is oxidized chiefly by xanthine oxidase, *in vivo*, by processes similar to those observed *in vitro*.

4-Mercaptopteridine is a substrate for xanthine oxidase but not for rabbit liver aldehyde oxidase. As mentioned earlier, the nature of the apparent major product detected in the urine of mice which received 4-mercaptopteridine and allopurinol remains unknown. If *S*-methylation could occur (such a methylation has been shown to occur for 6-mercaptopurine *in vivo* [31, 32]), a degradation pathway, similar to that observed in chemical systems [24], leading to

a pyrazine derivative, could be postulated to occur, *in vivo*.

The results of initial velocity studies (Table 6) and kinetic constant estimations (Table 7) show that various pteridines are capable of serving as substrates for mouse liver xanthine oxidase and strengthen the contention that this enzyme plays a role in pteridine oxidation, *in vivo*. The relative rates of oxidation of the 3 aminopteridines shown in Table 6 are similar to those estimated previously using milk xanthine oxidase [3], with the exception that 4-aminopteridine appears to be oxidized faster by the liver than by the milk enzyme. As can be seen from Table 6, the rates of oxidation of 2-hydroxypteridine and 2-mercaptopteridine are appreciably slower than their 4-hydroxy derivatives. 4-Mercaptopteridine is oxidized at a rate intermediate to that of 4-aminopteridine and 4-hydroxypteridine. The finding (Table 7) that the $K_m$ for 4-hydroxypteridine is greater than that of 2-amino-4-hydroxypteridine agrees with observations made by Krenitsky *et al.* [5] using milk enzyme.

It should be emphasized that in our studies of urinary metabolites of pteridine derivatives complete recovery of the administered pteridine (either unchanged or as metabolites) was not achieved. In this regard it is worth noting that extensive excretion of a pteridine derivative (triamterene) and its metabolites via biliary excretion has been reported [33] and loss of pteridines by this route could account for the relatively low recovery of pteridines in urine observed in some of our studies.

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