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STUDIES ON THE MODE OF OXIDATION OF PYRAZOLO(3,4-*d*)PYRIMIDINE BY ALDEHYDE OXIDASE AND XANTHINE OXIDASE*

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Abstract—Enzymic oxidation of the purine isomer pyrazolo(3, 4-*d*) pyrimidine to the xanthine isomer 4, 6-dihydroxypyrazolo(3, 4-*d*)pyrimidine is catalyzed by the metalloflavoproteins xanthine oxidase and aldehyde oxidase. The aldehyde oxidase-satalyzed reaction proceeds conventionally, utilizing molecular oxygen as electron acceptor. The xanthine oxidase-catalyzed reaction is anomalous in type, proceeding rapidly in the presence of artificial electron acceptors for the enzyme (phenazine methosulfate, 2, 6-dichlorophenolindophenol, ferricyanide), but extremely slowly with the acceptors molecular oxygen and cytochrome *c*. Anaerobic incubation of pyrazolo (3, 4-*d*) pyrimidine with enzyme results in substrate-induced reduction of the flavin moiety of aldehyde oxidase, but not in reduction of the flavin moiety of xanthine oxidase. The latter observation, together with the anomalous acceptor requirements, support the possibility that the xanthine oxidase-catalyzed reaction occurs by direct electron transfer to an external acceptor, by-passing the internal electron transport chain of the enzyme.

SEVERAL derivatives of pyrazolo(3, 4-*d*)pyrimidine (PP) have been the subject of biochemical, pharmacologic and clinical study. Compounds of particular interest in this series are 4-hydroxypyrazolo(3, 4-*d*)pyrimidine (4-HPP), a powerful xanthine oxidase inhibitor of value in the control of gout and other hyperuricemic states,[1] and 4-aminopyrazolo(3, 4-*d*)pyrimidine, an agent with significant antineoplastic activity in certain experimental tumor systems.[2,3]

Both 4-aminopyrazolo(3, 4-*d*)pyrimidine and 4-HPP are subject to 6-hydroxylation *in vivo*. The metabolic oxidation is of particular significance in the case of the latter compound because of evidence that the slowly excreted metabolite, 4, 6-dihydroxypyrazolo (3, 4-*d*) pyrimidine (4, 6-diHPP), is in part responsible for the pharmacologic activity of the drug.[4] The enzyme responsible for the hydroxylation is thought to be xanthine oxidase. *In vivo*, however, the rate of 6-hydroxylation of 4-HPP is rapid, while the hydroxylation of 4-HPP by xanthine oxidase *in vitro* is extremely slow.[5,6] The reasons for this disparity have not been fully elucidated, although recent studies from this laboratory indicate that the xanthine oxidase–catalyzed reaction proceeds rapidly enough *in vitro* to account for the metabolic conversion if an artificial electron acceptor is added to the system to act as a "carrrier" from the enzyme to the physiologic two-electron acceptor, molecular oxygen.[7,8] Little is known, however, about the possible

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role of acceptors other than oxygen in the reoxidation of reduced xanthine oxidase *in vivo*.

The synthesis of PP, the unsubstituted parent compound of the pyrazolo (3, 4-*d*)-pyrimidine series, was reported some years ago,[9] but its biological properties, unlike those of the 4-hydroxy and the 4-amino derivatives, have not been investigated. The present studies were initiated to determine whether PP possessed activity of potential pharmacologic interest as either a substrate or inhibitor of the purine-catabolizing enzymes aldehyde oxidase and xanthine oxidase. The compound was found to be a rapidly oxidized substrate for both enzymes, with the mode of oxidation by xanthine oxidase being of particular interest because of its similarity to the anomalous mode of oxidation of 4-HPP cited above. A description of these studies, some of which have been presented in preliminary form,[10] forms the body of this report.

MATERIALS AND METHODS

Materials. Phenazine methosulfate, estradiol-17β and xanthine were obtained from Calbiochem; menadione from Nutritional Biochemicals Corp.; 2, 6-dichlorophenol-indophenol from Eastman Kodak; Triton X-100 from Rohm and Haas; cytochrome *c*, Type III, from Sigma Chemical Co.; cellullose thin-layer chromatography plates from Mann Research Laboratories; nitrogen, research grade, from Matheson Co.; frozen mature rabbit liver from Pel-Freez Biologicals; catalase from Worthington Biochemical Corp. (Code CTS); and bovine milk xanthine oxidase from Boehringer (Code 15347) and from Worthington Biochemical Corp. (Codes XO and XOP). 4-HPP was generously supplied by Dr. G. B. Elion, Wellcome Laboratories; and PP and 4, 6-diHPP were synthesized as previously described.[9]

Enzyme purification. Rabbit liver aldehyde oxidase was purified by heat treatment, ammonium sulfate and acetone fractionation, and adsorption on and elution from calcium phosphate gel, as described by Rajagopalan *et al.*[11] Aldehyde oxidase activity was assayed spectrophotometrically by measuring the rate of conversion of 3′, 5′-dichloromethotrexate to 3′, 5′-dichloro-7-hydroxymethotrexate, as previously described.[12] The specific activity of the aldehyde oxidase preparation used in the present studies was 77 mμmoles of 3′, 5′-dichloromethotrexate oxidized per mg protein per min, representing a purification of approximately 80-fold over the activity of the supernatant fraction from the crude liver homogenate.

Commercial bovine xanthine oxidase was chromatographed on hydroxylapatite, as described by Fridovich.[13] The specific activity of the fractions used in the present experiments varied between 6 and 9 units per mg (as defined by the latter author) and the A_{280}/A_{450m_μ} ratio was between 5·8 and 6·0.

Enzyme assay methods—Xanthine oxidase. Serial ultraviolet absorption spectra of the enzyme-catalyzed conversion of substrate to product were obtained with a Cary 15 recording spectrophotometer. Studies of the substrate-induced spectral change in the flavin spectrum of xanthine oxidase were carried out under nitrogen, using Thunberg-type anaerobic cuvettes (Hellma, Codes 190 and 197) and were recorded with a Cary 15 recording spectrophotometer equipped with a 0 to 0·1 O.D. slide wire.

Enzyme assays at a single wave-length were carried out with a Gilford multiple sample absorbance recorder. Assays were performed at 25° in potassium phosphate buffer, pH 7·8, 0·05 M, containing EDTA, 10^{-5} M. For the substrates PP and 4-HPP, absorption spectra of the substrates and of the product (4, 6-diHPP) were first deter-

mined at the pH of the assay mixture, and the following wavelengths were selected as convenient for assay: For PP to 4, 6-diHPP, 240 mμ ($\Delta\epsilon$ = + 5550); for 4-HPP to 4, 6-diHPP, 285 mμ ($\Delta\epsilon$ = + 2060). The conversion of xanthine to uric acid was followed at 290 mμ.[14] Details of individual assay methods are given in the legends for figures and tables.

Enzyme assay methods—Aldehyde oxidase. Serial spectra of the enzyme-catalyzed conversion of substrate to product were obtained with a Cary 15 recording spectrophotometer. Difference spectra of the substrate-induced changes in the flavin spectrum of aldehyde oxidase were obtained with a Shimadzu MPS-50L recording spectrophotometer, using the − 0·1 to + 0·1 absorbance range.

Enzyme assays at a single wave-length were carried out with a Gilford multiple sample absorbance recorder. Assays were carried out at 25° in sodium phosphate buffer, pH 7·8, 0·04 M, containing Versene Fe-3, 0·005%. The oxidation of both substrates, PP and 4-HPP, was followed at 290 mμ. The values of changes in extinction at this wave-length, previously determined at the pH of the assay mixture, were as follows: PP to 4-HPP, $\Delta\epsilon$ = − 1770; 4-HPP to 4, 6-diHPP, $\Delta\epsilon$ = + 1200. Details of individual assay methods are given in the legends for figures and tables.

Protein concentration was determined by the biuret method.

RESULTS

PP as a substrate for xanthine oxidase

Studies with artificial electron acceptors. The ability of PP to act as a substrate for xanthine oxidase was initially determined by following the rate of enzyme-catalyzed reduction of a series of known artificial electron acceptors for the enzyme. With all the artificial acceptors examined, PP was found to be a rapidly oxidized substrate for xanthine oxidase, the reaction velocities observed being comparable to those seen with the physiologic substrate xanthine and with 4-HPP under the same conditions (Table 1). Like the latter substrate, however, but unlike xanthine, PP was almost devoid of ability to reduce the one-electron acceptor cytochrome *c*, unless an intermediate acceptor or "carrier" such as phenazine methosulfate was added to the system. With phenazine methosulfate as carrier, the rate of enzyme-catalyzed reduction of

TABLE 1. RATES OF XANTHINE OXIDASE–CATALYZED REDUCTION OF ELECTRON ACCEPTORS BY PYRAZOLO (3, 4-*d*) PYRIMIDINES AND BY XANTHINE*

Electron acceptor	Rate (μmoles acceptor reduced/mg enzyme protein/min)		
	Xanthine	PP	4-HPP
Potassium ferricyanide, 10^{-3} M	0·08	0·03	0·02
2, 6-Dichlorophenolindophenol, 10^{-4} M	0·20	0·16	0·29
Cytochrome *c*, 8×10^{-5} M	0·05	0·003	0
Cytochrome *c*, 8×10^{-5} M, plus phenazine methosulfate, 8×10^{-5} M	0·27	0·41	0·42

*Cuvettes contained potassium phosphate buffer, pH 7·8, 150 μmoles; EDTA, 0·03 μmoles; xanthine oxidase, 0·16 mg; substrate, 1 μmole; and acceptor at the concentration indicated, in a total volume of 3 ml. In experiments with cytochrome *c*, the reaction mixture also contained catalase, 25 units. Assays were performed under aerobic conditions at 25°. Wavelengths used for assay were as follows: Potassium ferricyanide, 420 mμ ($\Delta\epsilon$ = 10,820); 2, 6-dichlorophenolindophenol, 600 mμ ($\Delta\epsilon$ = 21,000); cytochrome *c*, 550 mμ ($\Delta\epsilon$ = 19,600).

cytochrome *c* by PP was extremely rapid, being almost twice that seen with xanthine as substrate (Table 1).

Serial spectrophotometric studies of product formation. The physiologic electron acceptor for xanthine oxidase is generally considered to be molecular oxygen,[15] and most xanthine oxidase–catalyzed reactions proceed at significant rates in the presence of atmospheric oxygen alone, without the addition of artificial acceptors or carriers. In the absence of a carrier species, however, the rate of xanthine oxidase–catalyzed hydroxylation of PP, as determined by serial spectrophotometric studies, was found to be extremely slow. In the experiment shown in Fig. 1, aerobic incubation of PP,

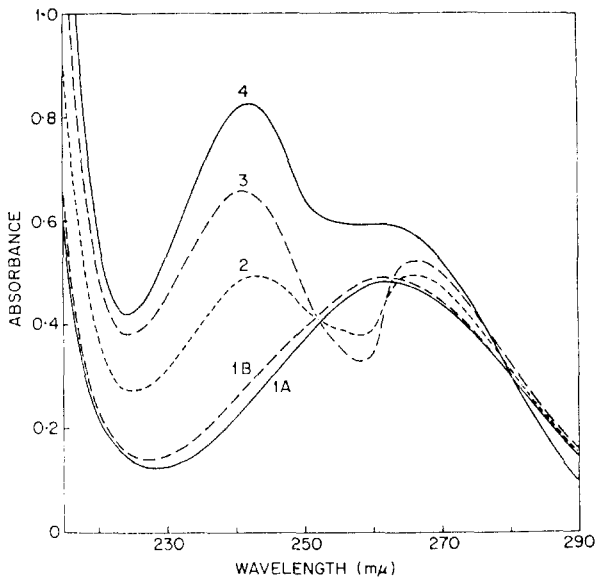

FIG. 1. Stimulation by phenazine methosulfate of the conversion of PP to 4, 6-diHPP by xanthine oxidase. The reference cuvette contained potassium phosphate buffer, pH 7·8, 150 μmoles; EDTA, 0·03 μmoles; and catalase, 25 units in a total volume of 3 ml. The sample cuvette contained the same constituents plus PP, 0·27 μmoles. At 0 min, xanthine oxidase, 0·08 mg, was added to both cuvettes and spectrum 1A was recorded. After aerobic incubation with enzyme at 25° for 2 hr 30 min, spectrum 1B was recorded. Phenazine methosulfate, 0·04 μmole was then added to both cuvettes and spectra recorded at 30 min, (2); 60 min, (3); and 2 hr (4). No further change in spectrum was detectable; spectrum 4 is identical with that of 4, 6-diHPP. Catalase, although present in this experiment, was not an essential component of the reaction mixture.

0·32 μmoles, in the presence of xanthine oxidase, 0·08 mg, for 2½ hr, resulted in only very slight spectral changes, indicating a utilization of approximately 0·02 μmoles of substrate (spectrum 1B). With the addition of a substoichiometric amount (0·04 μmole) of the artificial acceptor phenazine methosulfate,* however, the reaction proceeded rapidly to completion. The spectrum of the reaction product (Fig. 1, spectrum

*Phenazine methosulfate was used as acceptor in the serial spectrophotometric studies because the reduced form of this acceptor is rapidly reoxidized by air,[16] and thus does not contribute significantly to the ultraviolet absorption spectrum. In the experiment shown in Fig. 1, the rate of reduction of phenazine methosulfate in the sample cuvette slightly exceeds its rate of aerobic reoxidation, resulting in the drop in base-line observed over the region 250–265 mμ of spectra 2 and 3. Because of complete reoxidation of the acceptor by the completion of the experiment, this effect is not observed in spectrum 4.

4) was identical to that of 4, 6-diHPP. No reaction was observed if enzyme was omitted from the system.

Product identification by thin-layer chromatography. The identity of the reaction product as 4, 6-diHPP was confirmed by cellulose thin-layer chromatography of aliquots from the supernatant of the deproteinized reaction mixture, using synthetic 4, 6-diHPP as a reference compound [R_f = 0·36 in *n*-butanol : acetic acid : water (58 : 24 : 18) and 0·10 in *n*-butanol : water (86 : 14) with ammonia in the vapor phase].

Relative rates of oxidation of PP and of 4-HPP. Neither the chromatographic nor the serial spectrophotometric studies described above demonstrated the presence of intermediate monohydroxy reaction products, and it is not therefore possible to state with certainty whether the intermediate in the oxidation of PP to 6, 6-diHPP is the 4-hydroxy or the 6-hydroxy compound. It was felt that determination of the relative rates of oxidation of PP and of the two possible intermediates might be of value in elucidating this point, since if the rate of oxidation of one of the latter was found to be significantly slower than that of the parent compound, it could be eliminated from consideration as a participant in the overall reaction. Of the two possible monohydroxy intermediates, no reports of the synthesis of 6-hydroxypyrazolo (3, 4-*d*) pyrimidine could be located, and thus only 4-HPP was available for study. In the presence of phenazine methosulfate at the concentration used in the experiment shown in Fig. 1, the rate of xanthine oxidase–catalyzed oxidation of 4-HPP was found to be slightly faster than that of PP (Fig. 2, A and B); 4-HPP is therefore a possible intermediate in the reaction,

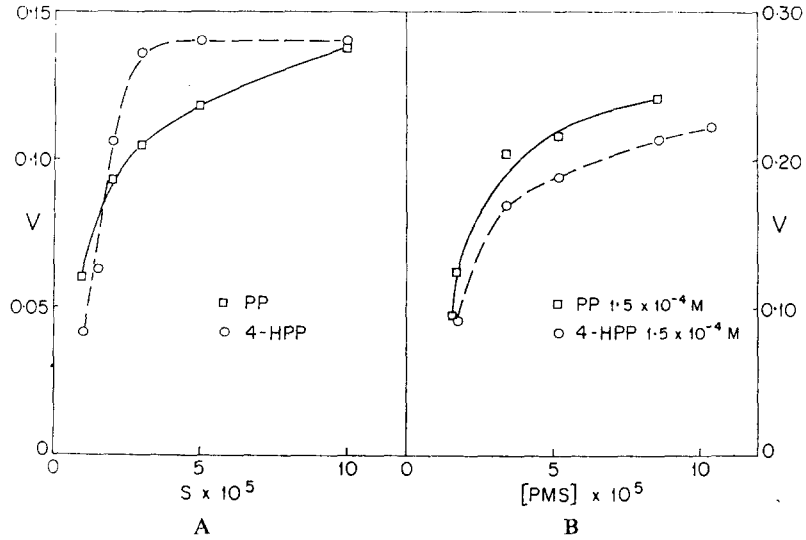

FIG. 2. A. (left) Effect of substrate concentration on rates of enzymic oxidation of pyrazolo (3, 4-*d*)-pyrimidines. Cuvettes contained potassium phosphate buffer, pH 7·8, 150 μmoles; EDTA, 0·03 μmole; phenazine methosulfate, 0·04 μmoles; xanthine oxidase, 0·08 mg; and substrate as indicated, in a total volume of 3 ml. V = μmoles substrate oxidized per mg enzyme protein per min.

B. (right) Effect of phenazine methosulfate concentration on rates of enzymic oxidation of pyrazolo-(3, 4-*d*) pyrimidines. Cuvettes contained potassium phosphate buffer, pH 7·8, 150 μmoles; EDTA 0·03 μmole; substrate, 0·45 μmoles; xanthine oxidase, 0·08 mg, and phenazine methosulfate as indicated, in a total volume of 3 ml. V = μmoles substrate oxidized per mg enzyme protein per min.

since its oxidation at a rate greater than that of the parent compound would prevent it from accumulating in the reaction mixture in concentrations sufficient for detection.

Effectiveness of PP in eliciting substrate induced changes in the flavin spectrum of xanthine oxidase. Conventional substrates for xanthine oxidase, when incubated with the enzyme under anaerobic conditions, bring about reduction of the internal electron transport chain of the enzyme; the reduction is detectable spectrophotometrically by following the reduction of the flavin moiety to the dihydro form.*,[17-19] The extremely low substrate activity of PP with molecular oxygen or cytochrome *c* as electron acceptors raised the possibility that transfer of electrons from this substrate to the conventional internal electron transport chain was unable to take place. When this possibility

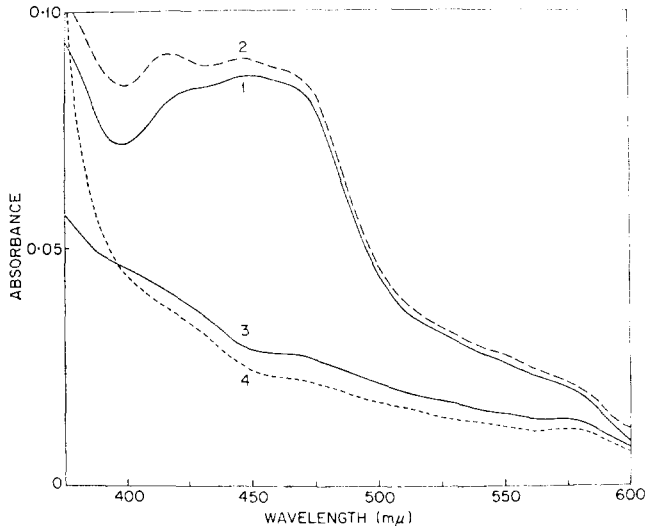

FIG. 3. Effect of anaerobic incubation with substrates on flavin spectrum of xanthine oxidase. Thunberg-type cuvettes contained potassium phosphate buffer, pH 7·8, 150 μmoles; EDTA, 0·03 μmole; and xanthine oxidase, 1·5 mg, in a total volume of 3 ml. Curve 1: no substrate. Curve 2: anaerobic incubation with PP, 0·5 μmole, for 90 min. Curve 3: anaerobic incubation with xanthine, 0·5 μmole, for 90 min. Curve 4: enzyme flavin reduced by addition of sodium dithionite.

was tested experimentally, by anaerobic incubation of PP with xanthine oxidase for periods up to 3 hr, it was indeed found that this substrate failed to bring about spectrophotometrically detectable alteration in the flavin spectrum; in control experiments with the same enzyme preparation, xanthine brought about "immediate" or "rapid phase" reduction of 32 per cent of the enzyme-bound flavin, proceeding to 92 per cent reduction over a period of 90 min (Fig. 3).

PP as an inhibitor of xanthine oxidase

As noted above, PP, although rapidly oxidized by xanthine oxidase when artificial

* The form of the enzyme flavin thought to participate in electron transfer is not the dihydro form, but a partially reduced or semiquinone form.[20] However, the two reduced forms are in equilibrium, so that, when substrate is present in excess, formation of the catalytically active semiquinone also gives rise to the spectrophotometrically detectable dihydro form.

electron acceptors are present in the system, undergoes only extremely slow oxidation with molecular oxygen alone as electron acceptor. In the absence of any acceptor other than oxygen, therefore, it was possible to study the ability of PP to act as an inhibitor of the xanthine oxidase–catalyzed electron transfer from the physiologic substrate xanthine to oxygen. Under these conditions, PP was found to be an effective inhibitor of the oxidation of xanthine, provided that it was added to the system before the addition of substrate. The degree of inhibition increased with the length of time of

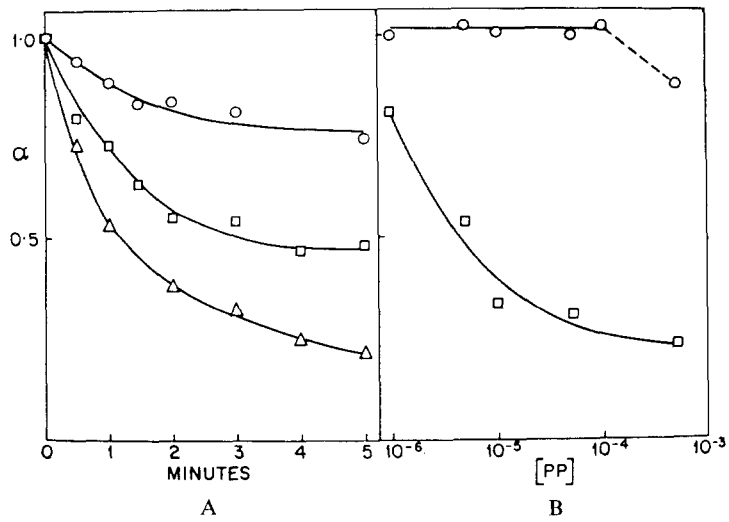

FIG. 4. A. (left) Effect of time of preincubation of PP and xanthine oxidase on the rate of oxidation of xanthine. Cuvettes contained potassium phosphate buffer, pH 7·8, 150 μmoles; EDTA, 0·03 μmoles; xanthine oxidase, 0·01 mg; and PP at the concentration indicated below, in a total volume of 3ml. After preincubation of enzyme and PP for the time indicated in the abscissa, xanthine, 0·3 μmole, was added and its rate of conversion to uric acid determined. α = observed rate as a fraction of rate obtained when xanthine and PP were added simultaneously. ○———○ PP, 10^{-6} M; □———□ PP, 5×10^{-6} M; △———△ PP, 10^{-5} M.

B. (right) Protection by substrate against inhibition of xanthine oxidase by PP. Cuvettes contained potassium phosphate buffer, pH 7·8, 150 μmoles; EDTA, 0·03 μmole; xanthine oxidase, 0·01 mg; xanthine, 0·3 μmole; and PP at the concentration indicated, in a total volume of 3 ml. α = observed rate as a fraction of rate obtained when PP was omitted. ○———○; PP and xanthine added to the reaction mixture simultaneously; □———□, PP incubated with enzyme for 3 min before the addition of xanthine.

pre-incubation of enzyme and PP (Fig. 4, A). No inhibition was noted except at extremely high concentrations when PP was added to the reaction mixture after the addition of xanthine (Fig. 4, B). The unusual features of the inhibition (i.e. the time-dependent increase in inhibition, and the protection by substrate) are similar to those described for the xanthine oxidase inhibitors 4-HPP[21] and purine-6-aldehyde.[22]

PP and 4-HPP as substrates for aldehyde oxidase

Mammalian liver aldehyde oxidase, a metalloflavoprotein which catalyzes the oxidation of aliphatic and aromatic aldehydes to the corresponding carboxylic acids,

has the ability in a number of species (notably rabbit and guinea pig) to catalyze the ring–hydroxylation of a variety of *N*-heterocyclic compounds. Rabbit liver aldehyde oxidase can catalyze the rapid oxidation of purine to 8-hydroxypurine,[23] and the much slower oxidation of hypoxanthine to xanthine;[23] unlike xanthine oxidase, however, the rabbit liver enzyme does not catalyze the oxidation of the latter compound to uric acid, although slow conversion of xanthine to uric acid has been reported with the enzyme from hog liver.[24]

Since PP and 4-HPP are isomers of purine and hypoxanthine, respectively, it was felt to be of interest to assess the ability of these compounds to serve as substrates for aldehyde oxidase. PP, on aerobic incubation with rabbit liver aldehyde oxidase, was rapidly converted to a compound with the absorption spectrum of 4-HPP (Fig. 5).

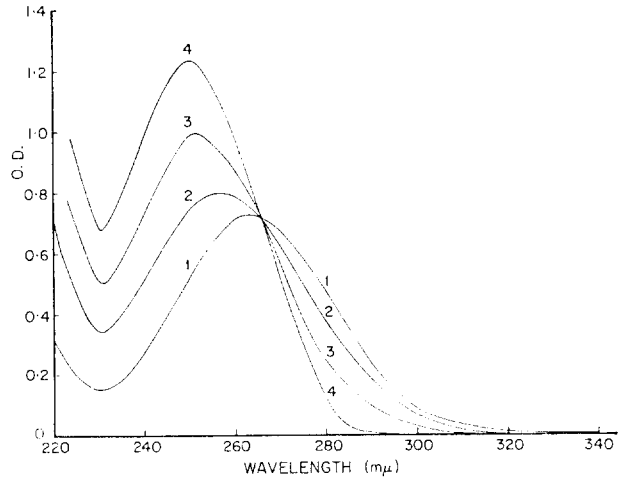

FIG. 5. Conversion of PP to 4-HPP by aldehyde oxidase. The reference cuvette contained sodium phosphate buffer, pH 7·8, 120 μmoles; Versene Fe-3, 18 μg, and rabbit liver aldehyde oxidase, 0·30 mg, in a total volume of 3 ml. The sample cuvette contained the same constituents plus PP, 0·42 μmoles. Spectra were recorded at 0 min, 1; 2 min, 2; 6 min, 3; and 13 min, 4.

With more extended periods of incubation, additional spectral changes were noted which suggested that the latter compound was undergoing further oxidation. Experments were therefore carried out with larger amounts of enzyme and with longer periods of incubation; these studies demonstrated that the initial oxidation product, 4-HPP, was indeed slowly converted by aldehyde oxidase to 4, 6-diHPP (Fig. 6). A quantitative comparison of the rates showed that, depending on the substrate concentrations used, the first hydroxylation step was from 3- to 6-fold more rapid than the second (Fig. 7). The identity of the two reaction products, 4-HPP and 4, 6-diHPP, was confirmed by cellulose thin-layer chromatography [R_f-values for 4-HPP: 0·57 in *n*-butanol : acetic acid : water (58 : 24 : 18) and 0·37 in *n*-butanol : water (86 : 14) with ammonia in the vapor phase. R_f values for 4, 6-diHPP were listed in a previous section].

The relative slowness of the second oxidation raised the possibility that this step might be catalyzed, not by aldehyde oxidase, but by a contaminating enzyme which had not been entirely removed during the enzyme fractionation procedures. It was

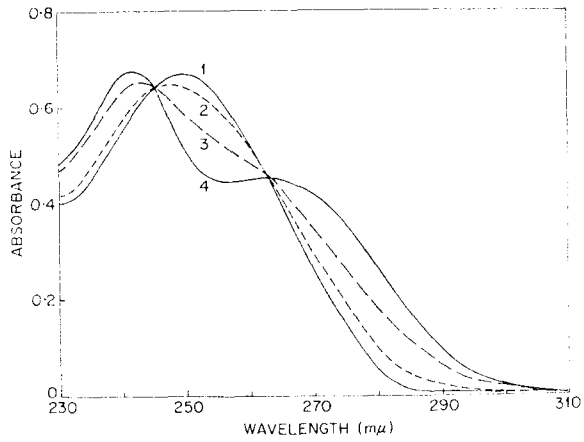

FIG. 6. Conversion of 4-HPP to 4, 6-diHPP by aldehyde oxidase. The reference cuvette contained sodium phosphate buffer, pH 7·8, 120 μmoles; Versene Fe-3, 18 μg; and rabbit liver aldehyde oxidase, 0·90 mg, in a total volume of 3 ml. The sample cuvette contained the same constituents plus 4-HPP, 0·22 μmole. Spectra were recorded at 0 min, 1; 1 hr, 2; 4 hr, 3; and 8 hr, 4.

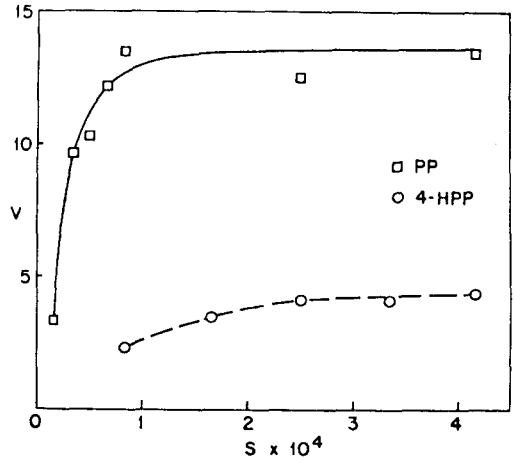

FIG. 7. Rates of oxidation of pyrazolo (3, 4-*d*) pyrimidines by rabbit liver aldehyde oxidase. Cuvettes contained sodium phosphate buffer, pH 7·8, 120 μmoles; Versene Fe-3, 18 μg; rabbit liver aldehyde oxidase, 1·5 mg; and substrate at the concentrations indicated. V = mμmoles substrate oxidized per mg enzyme protein per min. Assay procedures used were those described in Methods.

found, however, that both PP and 4-HPP, on aerobic incubation with aldehyde oxidase, brought about rapid substrate-induced reduction of the enzyme flavin (Fig. 8); furthermore, the conversion of 4-HPP to 4, 6-diHPP was slowed by the aldehyde oxidase inhibitors menadione, Triton X-100, and estradiol-17β (Table 2). The ability of 4-HPP to reduce the enzyme flavin, and the sensitivity of the oxidation to specific aldehyde oxidase inhibitors, indicated that aldehyde oxidase was indeed the catalytic agent responsible for the second hydroxylation step.

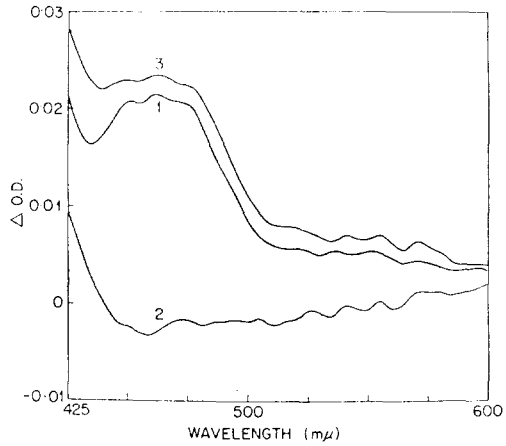

FIG. 8. Effect of anaerobic incubation with 4-HPP on the flavin spectrum of rabbit liver aldehyde oxidase. The reference cuvette contained sodium phosphate buffer, pH 7·8, 40 μmoles; Versene Fe-3, 6 μg; and rabbit liver aldehyde oxidase, 0·5 mg, in a total volume of 1 ml. The sample cuvette contained the same constituents plus 4-HPP, 0·5 μmole, in the side-arm. Both cuvettes were evacuated and flushed several times with nitrogen. 4-HPP was then tipped into the sample cuvette and the difference spectrum of the flavin region of aldehyde oxidase determined after 10-min incubation with substrate at 25° (spectrum 1). Difference spectra were then determined after admission of air to the sample cuvette (spectrum 2), and after the addition of sodium dithionite to the sample cuvette (spectrum 3).
Ordinate: Absorbance in reference cuvette minus absorbance in sample cuvette.

TABLE 2. EFFECT OF INHIBITORS ON THE CONVERSION OF 4-HPP TO 4, 6-diHPP BY RABBIT LIVER ALDEHYDE OXIDASE*

Inhibitor	Concentration of inhibitor required to reduce the rate of oxidation of 4-HPP to 50 per cent of control rate
Menadioneβ	4×10^{-6} M
Triton X-100	5×10^{-1} %
Estradiol-17β	4×10^{-5} M

*Cuvettes contained sodium phosphate buffer, pH 7·8, 120 μmoles; Versene Fe-3, 18 μg; 4-HPP, 1·5 μmoles; and rabbit liver aldehyde oxidase, 1·5 mg, in a total volume of 3 ml. Inhibitors were preincubated with enzyme for 2 min before the addition of substrate. Reaction rates were determined at 290 mμ, as described in the text.

DISCUSSION

The experiments described above are of interest both from the information they yield on the routes of enzyme-catalyzed catabolism of the pyrazolo (3, 4-*d*) pyrimidine series of compounds, and on the evidence they provide concerning routes of electron transfer by the metalloflavoprotein xanthine oxidase.

With reference to the first of these topics, the data indicate that both the purine-catabolizing enzymes xanthine oxidase and aldehyde oxidase possess the capacity to oxidize pyrazolo (3, 4-*d*) pyrimidines. Insofar as the parent compound, PP, is concerned, the oxidation by aldehyde oxidase is rapid and appears to be conventional in

nature, i.e. the reaction can utilize molecular oxygen as electron acceptor, and the oxidation is accompanied by reduction of the flavin prosthetic group of the enzyme. In these respects, the reaction is not dissimilar to the oxidation of the closely related isomer purine. These observations emphasize once again the extremely wide range of substrate specificity of rabbit liver aldehyde oxidase toward *N*-heterocyclic compounds first remarked some years ago by Knox.[25] The ability of compounds of the pyrazolo (3, 4-*d*) pyrimidine series to serve as substrates for this enzyme has not, to our knowledge, previously been noted.

Greater caution must be exercised regarding the significance of the 6-hydroxylation of 4-HPP by aldehyde oxidase. While the data indicate that the oxidation reaction described in the present paper is, in fact, catalyzed by aldehyde oxidase and not by a contaminating enzyme, the extreme slowness of the reaction would suggest that this pathway is unlikely to be of importance *in vivo*, even in species with high aldehyde oxidase activity such as rabbit and guinea pig. 4-HPP in this respect resembles its isomer hypoxanthine, which, although able to serve as a substrate for rabbit liver[23] and hog liver aldehyde oxidase,[24] is oxidized extremely slowly. It is likely therefore that the slow oxidation of 4-HPP by aldehyde oxidase, although of some interest with respect to the conformation of the substrate-binding site of the latter enzyme, is of little pharmacologic importance.

Inasmuch as xanthine oxidase is concerned, the mode of oxidation of PP by the latter enzyme does not resemble that of conventional substrates, but resembles in many respects the anomalous mode of oxidation we previously described for 4-HPP.[7,8] The extremely low substrate activity when molecular oxygen alone is used as an electron acceptor, the inability to reduce the flavin moiety of the enzyme, and the rapid rate of oxidation observed when an artificial acceptor is added, all indicate that PP, like 4-HPP, is unable to make effective use of the internal electron transport chain of the enzyme for rapid electron transfer directly to molecular oxygen. The simplest interpretation would appear to be that the mode of binding of these substrates to xanthine oxidase is sterically unfavorable for electron transfer from the 4- or 6-positions of the substrate to the initial acceptor species of the transport chain of the enzyme (probably a molybdenum atom tightly bound to the enzyme[26]). Transfer can, however, take place from the enzyme–substrate complex to any artificial acceptor of suitable oxidation–reduction potential added to the medium, since an external acceptor, not being a tightly-bound constituent of the enzyme protein, is not subject to steric limitations on receiving electrons from the substrate. With PP, however, the block appears not to be as complete as in the case of 4-HPP, since, with the former compound as substrate, rates with molecular oxygen and with cytochrome *c* are still detectable (Table 1 and Fig. 1), although extremely low when compared to the rates with artificial acceptors.*

That such a mechanism for electron transfer by xanthine oxidase may be of more than theoretical interest is indicated by the rapid rate of oxidation of 4-HPP which occurs *in vivo*.[28] The identity of a physiologic carrier species which would participate

*Further evidence that these considerations do not apply equally to all members of the pyrazolo-(3, 4-*d*) pyrimidine series is the observation that 4-aminopyrazolo (3, 4-*d*) pyrimidine is a conventional although rather slowly oxidized substrate of xanthine oxidase, which can utilize molecular oxygen as electron acceptor[27] and bring about substrate-induced reduction of the flavin prosthetic group of the enzyme.[8] It would appear therefore that the mode of electron transfer from these compounds to acceptor is not determined solely by the pyrazolo (3, 4-*d*) pyrimidine structure, but that the nature of substituents also influences the electron transfer process.

in this transfer to molecular oxygen *in vivo* is unknown; it is possible to eliminate from consideration, however, the one-electron carrier cytochrome *c*. With some substrates, including xanthine, cytochrome *c* can act as an effective electron acceptor for xanthine oxidase.[18, 29, 30] Cytochrome *c*, however, like molecular oxygen, is unable to act as an effective direct electron acceptor for either PP (Table 1) or for 4-HPP (Table 4 and Reference 7). This observation supports, although indirectly, the hypothesis of previous authors that the reduction of cytochrome *c* by xanthine oxidase is not a direct transfer, but first requires the reduction of molecular oxygen.[29, 30] Since these compounds cannot effectively utilize oxygen as a direct electron acceptor, the reduction of cytochrome *c* by this mechanism would thus not be anticipated. Electron transfer from xanthine oxidase to cytochrome *c in vivo* by some physiologic carrier species other than oxygen is a possibility, however, and has recently been suggested by Muraoka *et al.*[31] to explain the observation that crude xanthine oxidase, unlike the highly purified enzyme, is able to catalyze the reduction of cytochrome *c* under anaerobic conditions.

Another possibility exists to account for the rapid rate of oxidation of 4-HPP *in vivo*, i.e. that one or more enzyme or enzyme systems in addition to xanthine oxidase participates in the oxidation. Evidence has been cited above indicating that aldehyde oxidase probably does not function significantly in this connection, although it could well play a role in the oxidation of PP to 4-HPP. Strong and almost conclusive evidence that xanthine oxidase is, in fact, the enzyme responsible for the 6-hydroxylation of 4-HPP *in vivo* is the observation of Elion *et al.*[28] that a human subject lacking xanthine oxidase due to a genetic defect was unable to carry out this conversion, but instead excreted 4-HPP unchanged.

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