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XANTHINE OXIDASE ACTIVITY IN RAT BRAIN*

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SUMMARY

Xanthine oxidase activity was demonstrated in rat brain preparations by a spectrofluorometric method using pterine as a substrate. The activity of the enzyme in brain cytosol was 10 ± 4.3 nmoles/g protein per h (mean ± S.D.) as compared with an activity of 46.0 ± 24.9 μmoles/g protein per h in liver cytosol. Column chromatography of brain cytosol on Sephadex G-150 dextran resulted in elution of two peaks of 280 nm absorption. The first peak contained xanthine oxidase activity with a 174-fold increase in total activity over that measurable in the cytosol applied to the column, and an increase in specific activity to 2620.4 nmoles/g protein per h. The second peak contained an inhibitor to the enzyme assay. Similar chromatography of liver cytosol resulted in only a 1.54-fold increase in the yield of xanthine oxidase activity, a slight increase in specific activity to 54.9 μmoles/g protein per h and no detection of an inhibitor. The inhibitor separated from brain xanthine oxidase was identified as hypoxanthine by paper chromatography and its characteristic ultraviolet spectra. The brain enzyme had temperature and pH optima, apparent molecular weight, and kinetic properties similar to those of the liver enzyme. The presence of xanthine oxidase activity in brain tissue could contribute to the regulation of purine metabolism in the brain.

INTRODUCTION

Xanthine oxidase (EC 1.2.3.2) is a metalloflavoprotein which has the ability to catalyze the oxidation of a large number of substrates, among which are hypoxanthine and xanthine[1]. The enzyme has a wide tissue distribution, being most active in the soluble fraction of liver[2–4]. Several workers have been unable to detect xanthine oxidase activity in brain tissue[5–7]. However, the spectrophotometric assay which they used[8] is relatively insensitive[9]. A more sensitive method, based on fluorometry, has been reported recently[9–11].

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This study describes a modification of the spectrophotofluorometric assay for xanthine oxidase and its application to the demonstration of xanthine oxidase activity in rat brain preparations. Properties of that enzyme were then compared to those of rat liver xanthine oxidase.

EXPERIMENTAL PROCEDURE

Materials

Pterine (2-amino-4-hydroxypteridine) and horse spleen apoferritin were obtained from Calbiochem, isoxanthopterin (2-amino-4,7-dihydroxypteridine) from Aldrich, yeast alcohol dehydrogenase from Worthington, and Sephadex G-150 and G-200 from Pharmacia. Allopurinol (4-hydroxypyrazolo-(3,4-(+)-pyrimidine), xanthine, hypoxanthine, bovine albumin, bovine thyroglobulin and milk xanthine oxidase were purchased from Sigma.

Enzyme assay

Xanthine oxidase activity was determined by a modification of the fluorometric method originally described by Haining and Legan[9], which measures the production of a fluorescent product, isoxanthopterin, from pterine. An Aminco–Bowman spectrophotofluorometer, American Instrument Co. was used instead of the ratio fluorometer previously described permitting selection of discrete wavelengths for activation and for fluorescence. Pterine at a concentration of 10 μM was best activated by light of 360 nm wavelength, producing a small fluorescence peak at 440 nm. Isoxanthopterin was best activated at 345 nm, with a large fluorescence peak at 405 nm. When an activating wavelength of 345 nm was coupled to a fluorescence wavelength of 390 nm, pterine produced a fluorescence only 9% that of isoxanthopterin. All assays were thus carried out at these two wavelengths. An excess of substrate was used to assure constance of the fluorescence due to pterine. An external standard, 16 mM quinine sulfate in buffer, was used to set the spectrophotofluorometer to full-scale deflection before each reading. The volume of the assay mixture was 1.0 ml and consisted of 9 μM pterine, and enzyme preparation, 0.5 ml, in 0.04 M sodium phosphate buffer, pH 7.4. All assay reactions were pre-equilibrated and run in duplicate at a constant temperature water bath at 30 °C with continuous shaking. Fluorescence was read immediately and then again 30 min later. All reactions observed were first order for at least 1 h after initiation, save only for undiluted liver preparations. These were accordingly diluted 0.01-fold prior to assay. This system has the advantage that an initial zero-time reading, containing enzyme and substrate, is obtained. Both substrate and enzyme blanks were also run. The concentration of isoxanthopterin formed was calculated from the linear plot of fluorescence *versus* concentration obtained for standards from $1 \cdot 10^{-3}$ to 1 μg/ml.

Protein concentrations were determined in duplicate by the method of Lowry *et al.*[12], using crystalline bovine serum albumin as the standard. Final enzyme activities were expressed as μmoles (or nmoles) of isoxanthopterin formed/g protein per h.

Fractionation and partial purification

Male Wistar rats, 150–200 g body weight, previously maintained on an adequate diet were stunned by a blow to the neck. Brains and livers were rapidly removed

and washed in ice-cold isotonic saline. The cerebellum and lower brain stem were excised from each brain and discarded. The organs were blotted dry, weighed, and minced with scissors. All subsequent steps were carried out at 4 °C. 2 ml/g (wet weight) of 0.04 M sodium phosphate buffer, pH 7.4, were added, and each specimen was homogenized by ten passes in a Potter–Elvehjem glass homogenizer with a motor-driven teflon pestle. The crude homogenate was then fractionated by the method of Sackler[13], save only that three washes of buffer were used to ensure that the 480 × *g* sediment was free of lighter fractions. Nuclear, mitochondrial, microsomal and cytosol fractions were reserved for measurement of xanthine oxidase activity.

Brain and liver cytosol fractions were chromatographed on Sephadex G-150 dextran in a Pharmacia K25/45 column adapted for upward flow chromatography. 5-ml samples of cytosol (previously diluted one-fifth in the case of the liver) were applied to the column and eluted with 0.04 M sodium phosphate buffer, pH 7.4, at an average flow rate of 20 ml/h. Fractions of 10 ml were collected in a Gilson linear fractionator equipped for ultraviolet absorption recording. Eluted samples were assayed for xanthine oxidase activity and for protein concentration as described.

Study of enzyme properties

Enzyme-rich fractions obtained by chromatography were used in the measurement of pH and temperature optima and kinetics. Temperature optima were determined by running assay reactions at temperatures from 30 to 80 °C. Optimum pH was determined for each enzyme in assay reactions buffered by 0.04 M sodium phosphate buffer of pH 4.5 to 8.5. Kinetics of the brain and liver enzymes were studied by varying the pterine concentration from 2 to 9 μM. The kinetics of inhibition of the enzymes were determined with 10^{-7} M allopurinol and endogenous inhibitor (0.1–0.25 ml) recovered after chromatography of the brain cytosol. The kinetics of enzyme reaction and inhibition were analyzed by Lineweaver–Burk plots[14] and the inhibition constants, K_i, by Dixon plots[15] derived from regression analysis of the data.

Molecular weights of the brain and liver enzymes were estimated according to the method of Andrews[16] by gel filtration of the Sephadex G-150 fractions on a 2.3 cm × 45 cm column of Sephadex G-200. The column was equilibrated and eluted with 0.04 M sodium phosphate buffer, pH 7.4. Fractions of 5 ml were collected at a flow rate of about 20 ml/h. The molecular weight standards used were: bovine albumin, yeast alcohol dehydrogenase, milk xanthine oxidase, horse spleen apoferritin and bovine liver thyroglobulin. The eluted fractions were monitored spectrophotometrically for protein, and for activity in the case of the enzymes. The activity of alcohol dehydrogenase was determined by the method of Racker[17].

Paper chromatography of the endogenous inhibitor of xanthine oxidase was done by the method of Wyatt[18] using as solvent, isopropanol (65%, v/v), water and HCl (2.0 M). The spots were detected by ultraviolet light, and photographed by the method of Markham and Smith[19].

RESULTS

Xanthine oxidase activity was detected only in the cytosol fractions of tissue homogenates of brain and liver. The xanthine oxidase activity in twelve brains was

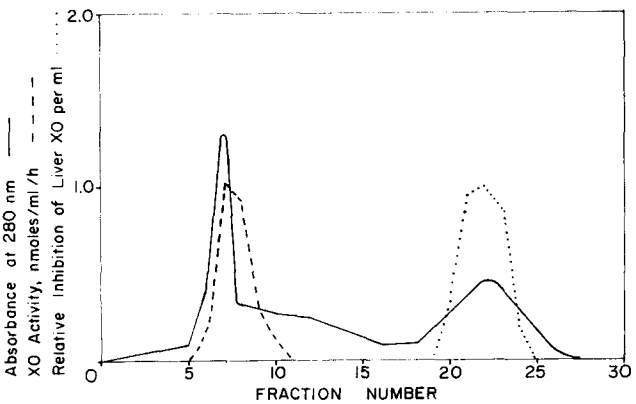


Fig. 1. Sephadex G-150 chromatography of brain cytosol. 5 ml of brain cytosol (7.74 mg of protein/ml) were placed on the column (2.5 cm × 45 cm) and eluted with 0.04 M sodium phosphate buffer, pH 7.4. Fractions of 10 ml were collected at a flow rate of about 20 ml/h. XO, xanthine oxidase.

10 ± 4.3 nmoles/g protein per h (mean ± S.D.). This contrasts with a much higher mean activity of 46.0 ± 24.9 μmoles/g per h obtained in six livers.

Sephadex chromatography of brain cytosol resulted in elution of two peaks of 280 nm absorption (Fig. 1). The first peak contained xanthine oxidase with a surprising 174-fold increase in total activity over that measurable in the cytosol applied to the column. The specific activity was likewise increased to 2620.4 nmoles/g protein per h (Table I). The second peak contained an inhibitor of xanthine oxidase activity. A histidine marker applied to the column was collected in fraction No. 22 of this peak. Sephadex chromatography of liver cytosol resulted in a much smaller

TABLE I

SEPHADEX G-150 COLUMN CHROMATOGRAPHY OF XANTHINE OXIDASE ACTIVITY OF BRAIN AND LIVER

Enzyme fraction	*Brain*				*Liver*			
	Total protein (mg)	*Total activity (nmoles/h)*	*Yield (-fold)*	*Specific activity (nmoles/g of protein per h)*	*Total protein (mg)*	*Total activity (nmoles/h)*	*Yield (-fold)*	*Specific activity (μmoles/g of protein per h)*
Cytosol	38.7	0.25		6.46	39.0	661.8		17.0
Eluate	16.6	43.50	174	2620.48	18.6	1021.7	1.54	54.9

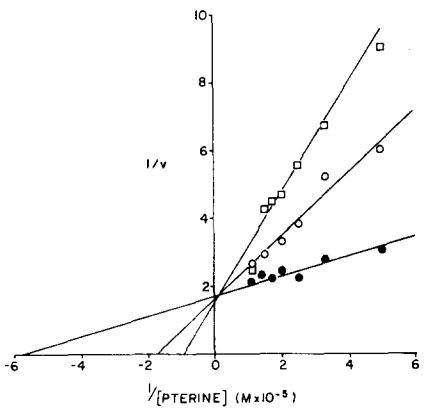
increase in xanthine oxidase specific activity, and only a 1.54-fold increase in yield. No inhibitor was detected in any of the liver fractions eluted. Milk xanthine oxidase applied to the column was recovered in fraction No. 7 corresponding to the peak elution of both brain and liver xanthine oxidase.

Properties of the enzyme

Optimal activities for brain and liver xanthine oxidase activity were observed at 55 and 50 °C, respectively. The brain enzyme showed a precipitous fall in activity

when heated past this point, with loss of all detectable activity by 75 °C. The liver enzyme was denatured above 55 °C. Maximal activity for the brain enzyme was found at pH 6.5 with little change in activity between pH 4.5 and 8.0. Beyond pH 8.0 however there was a rapid drop in activity, with only 50% of maximum activity remaining at pH 8.5. Optimal activity for liver xanthine oxidase was obtained between pH 6.0 and 7.0, with rapid decrements in activity above and below these values.

The Michaelis–Menten constants (K_m) of pterine for brain and liver xanthine oxidase were $1.7 \cdot 10^{-6}$ and $0.96 \cdot 10^{-6}$ M, respectively. Allopurinol (10^{-7} M) produced competitive inhibition of both the brain (Fig. 2) and liver enzyme (Fig. 3). When



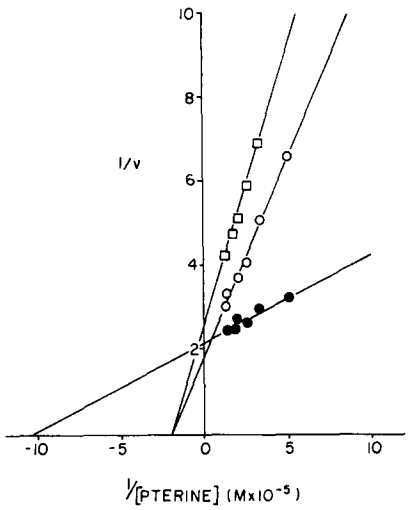


Fig. 2. Lineweaver–Burk plot of partially purified brain xanthine oxidase activity (●—●). Inhibition by 10^{-7} M allopurinol (□—□) or 250 µl of endogenous inhibitor eluted from Sephadex G-150 chromatography of brain cytosol (○—○).

Fig. 3. Lineweaver–Burk plot of partially purified liver xanthine oxidase activity (●—●). Inhibition by 10^{-7} M allopurinol (□—□) and 250 µl of endogenous inhibitor eluted from Sephadex G-150 chromatography of brain cytosol (○—○).

250 µl of the second 280-nm absorbance peak was substituted for buffer in the reaction mixture competitive inhibition of the brain (Fig. 2) and liver (Fig. 3) enzymes was demonstrated.

Activity of brain xanthine oxidase with hypoxanthine and xanthine was demonstrated by the spectrophotometric assay[8]; the Michaelis–Menten constants for hypoxanthine and xanthine were $2.2 \cdot 10^{-6}$ and $8.8 \cdot 10^{-5}$ M, respectively.

Further purification of the brain enzyme by precipitation with $(NH_4)_2SO_4$ between 35 and 60% saturations[4] did not result in a further increase in total enzyme activity, but resulted rather in a 48.9% recovery of the enzyme activity and a 1.46-fold increase in specific activity. None of the following enzymatic properties measured were changed by $(NH_4)_2SO_4$ precipitation: pH and temperature optima, K_m for pterine and competitive inhibition by the endogenous inhibitor.

Both the brain and liver enzymes were eluted in the same fraction as milk xanthine oxidase when chromatographed on Sephadex G-200. The molecular weight for both the brain and liver enzymes estimated from the calibration line obtained with the standards was approx. 275 000.

Properties of the inhibitor

Dialysis of brain cytosol against 0.04 M sodium phosphate buffer, pH 7.4, for 18 h resulted in removal of some of the inhibitor as evidenced by an increase in xanthine oxidase activity from 3.8 to 12.0 nmoles/ml per h. A much larger increase in the specific activity of xanthine oxidase from 10.2 to 2027 nmoles/per h was obtained when brain cytosol was precipitated by acetone followed by resuspension in buffer. The inhibitory activity recovered in the eluates following Sephadex column chromatography was not affected by acidification with 0.5 M H_2SO_4, followed by boiling for 1 h.

Ultraviolet spectrophotometry of the peak inhibitory fraction eluted from the column revealed a maximum absorption at 250 nm and a minimum at 226 nm (Table II). Acidification of the inhibitor fraction decreased the wavelengths of maxi-

TABLE II

ULTRAVIOLET ABSORPTION MAXIMA AND MINIMA (A) OF THE INHIBITORY FRACTION OF XANTHINE OXIDASE ELUTED FROM SEPHADEX G-150 CHROMATOGRAPHY OF BRAIN CYTOSOL AND (B) OF HYPOXANTHINE

The absorbance spectra of the endogenous inhibitor and of hypoxanthine $1 \cdot 10^{-4}$ M dissolved in 0.04 M sodium phosphate buffer were determined before and after the addition of acid (0.1 ml of 3 M HCl), and base (0.1 ml of 6.6 M NaOH) to 1 ml of inhibitor or hypoxanthine. The absorbancee values obtained after the addition of the acid or base were corrected for the 10:11 dilution.

Compound	*pH of the solution*	*Absorbance*			
		Maximal		*Minimal*	
		Wavelength (nm)	*A*	*Wavelength (nm)*	*A*
(A) Inhibitor	7.4	250	0.980	226	0.332
(1 ml)	1.0	249	0.955	224	0.500
	13.2	262	0.860	233	0.346
(B) Hypoxanthine	7.4	250	1.050	222	0.250
($1 \cdot 10^{-4}$ M)	1.0	248	1.020	215	0.188
	13.2	262	1.080	235	0.379

mum and minimum absorbance, whereas addition of base increased them. These findings suggested that the inhibitor found in the brain was hypoxanthine[20]. The inhibitor was further identified as hypoxanthine by its similar mobility on paper chromatography (Fig. 4). Hypoxanthine applied to the Sephadex G-150 column eluted in the same fraction (No. 22) as the endogenous inhibitor.

The inhibitory fraction was shown to be a substrate of both purified brain and milk xanthine oxidase in the spectrophotometric assay[8]. The total increase in absorbance at 290 nm/ml of peak inhibitory fraction reached after allowing the reaction to go to completion as compared with that obtained/μmole of hypoxanthine is shown in Table III. The inhibitory fraction did not inhibit initial rates of uric acid formation

Fig. 4. Paper chromatograph of the endogenous inhibitor and of oxypurine markers. (A) 100 μl of endogenous inhibitor. (B) 16 μg of hypoxanthine. (C) 20 μg of xanthine. The sites of application of the spots are indicated by the letter O. The solvent used was isopropanol (65%, v/v), water and HCl (2.0 M) and the chromatography was run for 24 h. The spots were detected by their absorption in ultraviolet light.

from either xanthine or hypoxanthine catalyzed by partially purified brain or milk xanthine oxidase.

Competitive inhibition of both the brain and liver enzymes was demonstrated with genuine hypoxanthine. The inhibition constant, K_i, for hypoxanthine was $1.1 \cdot 10^{-6}$ M for the brain enzyme and $2.5 \cdot 10^{-6}$ M for the liver enzyme. In the kinetic studies with the endogenous inhibitor, the volumes of endogenous inhibitor corresponding to the inhibition constant, K_i, were 20 μl and 35 μl per 1 ml of reaction

TABLE III

TOTAL INCREASE IN $A_{290\ nm}$ OBTAINED FOLLOWING OXIDATION OF ENDOGENOUS XANTHINE OXIDASE INHIBITOR AND HYPOXANTHINE CATALYZED BY PURIFIED BRAIN AND MILK XANTHINE OXIDASE

The reaction mixture, 3 ml, contained 0.14 M Tris–HCl buffer, pH 8.1, 0.7 mg albumin; 0.13 μmole/min xanthine oxidase or 0.15 μmole/min milk xanthine oxidase (Worthington); and either endogenous inhibitor, 1 ml or hypoxanthine $1.3 \cdot 10^{-6}$ M. During a 30 min incubation period at 30 °C the reaction was followed to completion with a DU Beckman spectrophotometer and an automatic Gilford recorder. The increase in absorbance at 290 nm was corrected for small changes observed in the absence of substrate.

Xanthine oxidase	*Endogenous inhibitor (per ml)*	*Hypoxanthine (per μmole)*
Brain	1.09	10.60
Milk	1.01	10.04

mixture for the brain and liver enzymes, respectively. The concentration of hypoxanthine in the inhibitor fraction was determined to be $9.3 \cdot 10^{-5}$ M from the absorbance data (Table III). Therefore the volumes found above correspond to concentrations of hypoxanthine of $1.9 \cdot 10^{-6}$ M and $3.3 \cdot 10^{-6}$ M, respectively, which closely approximate the K_i values determined for hypoxanthine.

The recovery of hypoxanthine from the Sephadex G-150 chromatography of brain cytosol, corresponding to 2.5 g of brain wet weight, was estimated to be 1.9 μmoles based on the absorption of the eluted fractions at 250 nm; this is equivalent to a hypoxanthine concentration of 0.75 μmole (or 103 μg) per g of brain wet weight.

DISCUSSION

Xanthine oxidase activity has been found in a variety of tissues, but most workers have failed to find activity in brain[5–7]. In the present study, xanthine oxidase activity was found in brain tissue by use of a modified sensitive spectrofluorometric method with pterine as a substrate. Previously Villela[21] had reported xanthine oxidase activity in low speed supernatant fractions of brain using a colorimetric method. However, the activity which he obtained was 1000-fold greater than that found here, and experiments in this laboratory using his method failed to yield any measurable activity. Numerous other reports of xanthine oxidase activity in liver and other tissues, but failure to detect activity in brain, are not surprising in view of the 21:1 ratio we found between the specific activity of partially purified liver as compared with brain xanthine oxidase and the finding of an endogenous inhibitor of the enzyme assay in brain but not liver. Brain xanthine oxidase, like the liver enzyme, was found in the soluble fraction of the homogenate. The similar temperature and pH optima, apparent molecular weight, Michaelis–Menten constants for pterine as a substrate, and competitive inhibition by allopurinol and the endogenous inhibitor found in brain, suggests that the brain enzyme is functionally identical to the known xanthine oxidase of liver.

The endogenous inhibitor of the xanthine oxidase assay found in brain tissue and separated from the enzyme by Sephadex G-150 chromatography was shown to be oxidized to uric acid by the enzyme, and identified as hypoxanthine by paper chromatography, and by its characteristic absorption spectra at neutral, acid and basic pH[20]. The Michaelis–Menten constant of $2.2 \cdot 10^{-6}$ M found for brain xanthine oxidase with hypoxanthine as a substrate is of similar magnitude as the constants of $1.7 \cdot 10^{-6}$ and $0.9 \cdot 10^{-6}$ M found for the brain and liver enzymes, respectively, with pterine as a substrate. The comparable affinity of these substrates for the enzyme is compatible with the strong inhibitory effect which we observed for endogenous hypoxanthine on the oxidation of pterine by xanthine oxidase.

The estimated hypoxanthine concentration of 103 μg/g in the rat brain in the present study is of comparable magnitude to oxypurine (xanthine *plus* hypoxanthine) concentrations ranging between 40 and 140 μg/g found by the enzymatic method in chicken tissues other than brain[22]. To our knowledge oxypurine concentrations in the brain have not been previously reported, although concentrations of oxypurines in the cerebrospinal fluid of normal man have been found to be 1.3 μg/ml (ref. 23).

Recent work[5] has been aimed at clarifying the mechanisms involved in regulation of uric acid levels in cerebrospinal fluid. The basic assumption has been that

uric acid is not produced in nervous tissue, due to lack of xanthine oxidase, and therefore must be transported into the cerebrospinal fluid from the blood. The discovery of xanthine oxidase activity in rat brain implies that uric acid found in cerebrospinal fluid may arise directly from degradation of purines in the central nervous system. The presence of xanthine oxidase in brain tissue could contribute to the regulation of purine metabolism in the manner already demonstrated for other tissues.

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