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XANTHINE OXIDASE-MEDIATED OXIDATION OF EPINEPHRINE*

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Abstract—It has been reported [M. Oka, *J. pharm. Soc. Japan* **57**, 566 (1961)] that epinephrine is capable of stimulating the oxidation of purines by xanthine oxidase (X.O.). The present investigation shows that such "stimulation" is, in fact, attributable to concomitant oxidation of epinephrine as well as of the purine substrate (hypoxanthine) by X.O. preparations. X.O. does not oxidize epinephrine under the conditions employed in the absence of an oxidizable substrate such as hypoxanthine. The oxidation of epinephrine, in the presence of X.O. and hypoxanthine is inhibited strongly by allopurinol [4-hydroxypyrazolo-(3,4-d)pyrimidine]. Spectroscopic and chromatographic studies indicate that the product of epinephrine oxidation in the X.O. system is adrenochrome (2,3-dihydro-3-hydroxy-*N*-methylindole-5,6-quinone). Oxidation of 2-aminopteridine, 4-aminopteridine and 4-hydroxypteridine by X.O. also induced epinephrine oxidation. In the presence of 4-amino-6,7-dimethylpteridine and 2-amino-4-methylpteridine, which are not effective substrates for X.O., appreciable oxidation of epinephrine was not observed.

EPINEPHRINE has been reported[1] to stimulate the activity of xanthine oxidase (X.O.; xanthine: O_2 oxidoreductase, EC 1.2.3.2). We recently have been engaged in studies of the oxidation of pteridines and related heterocyclic compounds by X.O. and decided to investigate whether epinephrine and other pharmacologically active substances could affect the rate or pathway of oxidation of such compounds by this enzyme. It was found that epinephrine does not stimulate X.O. activity but rather that the "stimulation" reported previously is the result of concomitant oxidation of epinephrine and an oxidizable heterocyclic substrate by the enzyme. This communication describes our studies on the oxidation of epinephrine by X.O. preparations.

EXPERIMENTAL

Commercial xanthine oxidase preparations were obtained from the Worthington Biochemical Company (Code X.O.), Freehold, N.J. and from Nutritional Biochemicals Company, Cleveland, Ohio. Specific activities of the enzyme preparations used ranged from 0·08 to 0·13 units per mg of protein (1 unit = 1 μmole hypoxanthine oxidized/liter/min). Xanthine oxidase also was prepared from fresh raw milk (obtained through the cooperation of the Department of Animal and Dairy Science, University of Vermont) by the method of Ball.[2] Studies were carried out with the enzyme prepared

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by Ball's method because this type of preparation was employed by Oka[1] in his study of the effects of epinephrine on xanthine oxidase activity. Hypoxanthine and allopurinol were purchased from the Aldrich Chemical Company, Milwaukee, Wis. All pteridine derivatives were prepared in this laboratory by published procedures; the references to these synthetic procedures are given in the appropriate table. Adrenochrome and adrenolutin were prepared from epinephrine by the methods of Heacock *et al.*;[3,4] for the preparation of adrenochrome, the filtration through Dowex was carried out under nitrogen. L-Epinephrine bitartrate was obtained from Winthrop Laboratories, New York, N.Y. Sephadex G-25 was obtained from Pharmacia Fine Chemicals, Piscatawnay, N.J.

Paper chromatography was employed as an aid in identifying the product of epinephrine oxidation by X.O.; Whatman No. 1 paper prewashed with distilled water was used throughout. Chromatography was performed by the ascending method using butanol–acetic acid–water (12:3:5) or 2% acetic acid as solvents. Difficulty was encountered in visualizing adrenochrome in low concentrations on paper because this compound did not fluoresce under ultraviolet light (254 mμ); consequently additional treatment of the paper was required to detect the adrenochrome spots. Drying of the chromatogram in air with application of heat (hair dryer) results in rapid development of a yellow-green fluorescent spot at the R_f value expected for adrenochrome; this fluorescence is probably due to adrenolutin, a substance which can be formed readily by rearrangement of adrenochrome.[5] Adrenochrome could also be visualized as a blue spot by application of a ferricyanide chromogenic reagent to the paper.[3]

Rates of oxidation of epinephrine by X.O. in the presence of various heterocyclic substrates were studied using enzyme concentrations sufficient to oxidize 20 μmoles hypoxanthine/l./min (20 units/l.). Reaction velocities were estimated from the initial straight line portion of the plots of change in optical density versus time. Ultraviolet absorption measurements were made with a Perkin–Elmer model 202 spectrophotometer equipped with a sample chamber with a thermostat maintained at 37°. Gel filtration studies were carried out using a column (1 × 20 cm) of Sephadex G-25 equilibrated with phosphate buffer (pH 7·4) for 5 hr at 4°. Elution of the enzyme was performed using the same buffer.

RESULTS

A pink coloration was observed to develop rapidly when epinephrine was incubated with a pteridine substrate or with hypoxanthine in the presence of X.O. The appearance of such a color suggested that something more than simple stimulation of X.O. activity was occurring in the presence of epinephrine. The calculated optical density change at 290 mμ for complete conversion of hypoxanthine (5×10^{-5} M) to uric acid is 0·61; in the absence of epinephrine, this optical density change was produced by X.O. in 2–3 min. However, when epinephrine was added to the system, the optical density change (1·10) at 290 mμ over the same period of time was significantly greater than that anticipated for complete conversion of hypoxanthine to uric acid. The disparity between the observed and anticipated optical density change in the presence of epinephrine, together with the color development in the reaction cuvette, indicated that epinephrine was undergoing a transformation along with hypoxanthine in the presence of X.O. The rate of hypoxanthine oxidation in the presence of epinephrine, corrected for spectroscopic changes due to oxidation of epinephrine itself, was not

increased over the rate that was observed in the absence of epinephrine. Examination of the absorption spectrum of the reaction mixture in the visible range showed a maximum at 485 mμ. Adrenochrome (2,3-dihydro-3-hydroxy-*N*-methylindole-5,6-quinone) is known to be produced from epinephrine by a variety of oxidizing agents;[5] this substance gives a pink color in aqueous solutions and shows a maximum in the visible region at 485 mμ. Conversion of epinephrine to adrenochrome could result in the anomalous rise in optical density at 290 mμ seen with the X.O. system in the presence of epinephrine, since adrenochrome exhibits considerably higher absorption in this region of the ultraviolet spectrum than does epinephrine.[6] We therefore proposed that epinephrine was being oxidized to adrenochrome in the xanthine oxidase system; comparison of the absorption spectra of the oxidation product and of chemically prepared adrenochrome (Fig. 1) tends to support this proposal. Thus it appears that X.O. is capable of inducing the oxidative cyclization of epinephrine to

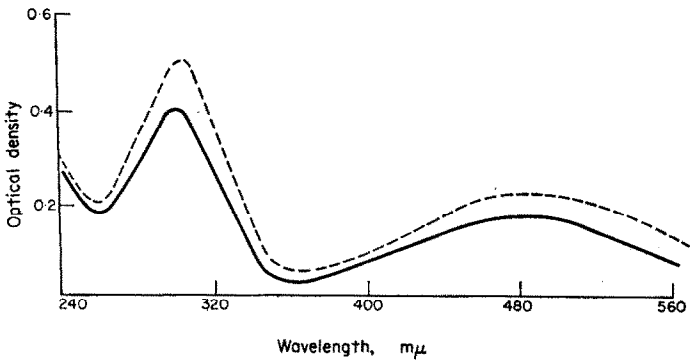

FIG. 1. Absorption spectra of epinephrine oxidation product (– – –) and adrenochrome (———). The spectrum of the epinephrine oxidation product was measured 3 min after incubation of epinephrine (5×10^{-5}M) with hypoxanthine (5×10^{-5}M) and X.O. (25 units/l.) in phosphate buffer (pH 7·4). The blank cuvette contained hypoxanthine, X.O. and buffer, but no epinephrine. The spectrum of adrenochrome ($4{\cdot}5 \times 10^{-5}$M) was measured at pH 7·4.

adrenochrome. To check the identity of the product of epinephrine oxidation in the X.O. system, we subjected the reaction mixture to chromatographic analysis. The results of the chromatographic studies are shown in Table 1; the data in this table support the assignment of the adrenochrome structure to the product of epinephrine oxidation.

When epinephrine was incubated with X.O. in the absence of hypoxanthine or with hypoxanthine in the absence of X.O., neither development of a pink color nor increase in optical density at 485 mμ was observed (Table 2). Similarly, no oxidation of epinephrine was observed when uric acid (the end product of hypoxanthine oxidation) was added to the catecholamine in the presence or absence of enzyme. Heating of X.O. for 10 min at 90° completely destroyed the ability of the enzyme to transform epinephrine. 4-Hydroxypyrazolo(3,4-*d*) pyrimidine (allopurinol) is a potent inhibitor of X.O.;[7] incubation of epinephrine with X.O. and hypoxanthine in the presence of allopurinol resulted in very little increase in optical density at 485 mμ. These results indicate that oxidation of hypoxanthine or a similar substrate is required in order for X.O. to induce adrenochrome formation from epinephrine. Hydrogen peroxide, a

TABLE 1. CHROMATOGRAPHIC DATA ON OXIDATION OF EPINEPHRINE BY XANTHINE OXIDASE

Substance	R_f*	Fluorescence†
Epinephrine	0·45 (0·83)	none
Adrenochrome	0·57 (0·89)	none
Adrenolutin	0·78 (0·41)	pink
Hypoxanthine	0·47 (0·67)	blue
Uric acid	0·31 (0·52)	blue
Epinephrine oxidation product†	0·57 (0·89)	none

* Paper chromatography was performed using butanol–acetic acid–water (12:3:5) as solvent; values given in parentheses were obtained using 2% acetic acid as solvent. All spots were dried in a closed container under nitrogen before development.

† Viewed at 254 mμ.

‡ For this chromatographic determination, X.O. (40 units/l.), hypoxanthine (0·03 mg) and epinephrine (0·1 mg) were incubated in pH 7·4 phosphate buffer at 37° for 5 min. The total reaction volume was 1·0 ml. At the end of the incubation period, 10 μl of the reaction system was spotted on Whatman No. 1 chromatography paper.

TABLE 2. OXIDATION OF EPINEPHRINE IN THE PRESENCE OF XANTHINE OXIDASE

System*	Δ O.D.$_{485}$/2 min
Complete	0·21
− Hypoxanthine	0·00
− X.O.	0·00
+ X.O. heated†	0·00
+ Allopurinol‡	0·02
+ Catalase§	0·22
Preincubation (5 min)‖	0·21
(20 min)	0·22
(50 min)	0·12
(120 min)	0·00

* The complete system consisted of X.O. (20 units/l.), hypoxanthine (5×10^{-5}M) and epinephrine (1×10^{-4} M) in phosphate buffer (0·067 M, pH 7·4); total reaction volume was 3·0 ml. All studies were performed at 37°.

† Enzyme in buffer solution was heated at 90° for 10 min, cooled to 37° and then treated with hypoxanthine and epinephrine.

‡ Enzyme was preincubated for 5 min with allopurinol (1×10^{-4} M).

§ Catalase (210 units) was added to the reaction mixture before addition of hypoxanthine and epinephrine.

‖ Figures in parentheses represent elapsed time in minutes, after oxidation of hypoxanthine to uric acid was completed, before addition of epinephrine to the reaction cuvette.

strong oxidizing agent, is produced during the course of oxidation of various substrates by X.O. To check whether hydrogen peroxide is, in some manner, responsible for epinephrine oxidation by X.O., we added a large excess of catalase to the reaction mixture; the presence of catalase did not affect appreciably the oxidation of epinephrine by X.O. (Table 2). Addition of a large excess of hydrogen peroxide to epinephrine also did not result in oxidation of the catecholamine under the conditions used in this study.

As shown in Table 3, there was no consistent stoichiometric relationship between the amount of uric acid produced by the X.O. system and the amount of epinephrine oxidized. Thus oxidation of hypoxanthine in the range 10^{-5}–10^{-6}M, by the enzyme was accompanied by production of 3–6 moles adrenochrome per mole of hypoxanthine oxidized; however, at higher concentrations of purine substrate the relative yield of adrenochrome decreased sharply. Further studies on the quantitative relationship between oxidation of hypoxanthine and other substrates in the X.O. system and oxidation of epinephrine are in progress.

TABLE 3. RELATIONSHIP BETWEEN URIC ACID FORMATION AND ADRENOCHROME FORMATION IN THE XANTHINE OXIDASE SYSTEM*

Conc. of hypoxanthine (μmoles/l.)	Uric acid formed (μmoles/l.)	Adrenochrome formed (μmoles/l.)
0	0	0
1×10^{-6}	1·1	5·0
2×10^{-6}	2·0	12·5
5×10^{-6}	5·0	20·0
1×10^{-5}	10·0	33·0
5×10^{-5}	50·0	50·0
1×10^{-4}	100·0	50·0

* Hypoxanthine (at the indicated concentrations) was incubated with X.O. (20 units/l.) and epinephrine (1×10^{-4} M) at pH 7·4 and 37°. Adrenochrome formation was measured by following the increase in optical density at 485 mμ until detectable oxidation of epinephrine ceased (approximately 5 min). An extinction coefficient of 4×10^3 was used to calculate the concentration of adrenochrome formed in the system. Formation of uric acid was measured by observation of optical density increase at 290 mμ in the absence of epinephrine. (In all cases uric acid formation was complete within 2–3 min.)

In order to determine whether simultaneous oxidation of hypoxanthine was required in order for epinephrine to be oxidized by X.O., we permitted complete oxidation of hypoxanthine to uric acid to occur and then added epinephrine at various times after oxidation of the purine substrate was complete. As shown in Table 2, addition of epinephrine at times up to 20 min after complete oxidation of hypoxanthine did not result in significant reduction of adrenochrome formation from that observed when epinephrine was present at the time when hypoxanthine oxidation was taking place. Epinephrine-oxidizing capacity did decrease sharply when the X.O.

system was incubated for longer periods of time before addition of epinephrine, but the significance of this is difficult to evaluate in view of the observation that the ability of X.O. to oxidize hypoxanthine also decreases considerably (approximately 50 per cent loss of activity) when the enzyme is incubated at 37° for such relatively long periods before addition of this substrate. It appears then that oxidation of hypoxanthine by X.O. results in an "activation" of the enzyme system for epinephrine oxidation and that the "activated" enzyme has a reasonably high degree of stability. Treatment of the activated enzyme with allopurinol did not result in inhibition of epinephrine oxidation, an observation which indicates that catecholamine oxidation *per se* is not mediated by the same enzymatic process as that involved in hypoxanthine oxidation. Heating of the activated enzyme preparation before addition of epinephrine destroyed its ability to oxidize the catecholamine. To examine the possibility that free metal ions (which conceivably might be released from the enzyme during the activation process) were responsible for epinephrine oxidation, we added EDTA (10^{-4}M) to the activated enzyme before adding epinephrine. The addition of EDTA was without effect on epinephrine oxidation.

Experiments were carried out to determine whether the epinephrine-oxidizing capacity of the activated X.O. system is retained when relatively low molecular weight substances are separated from the system by gel filtration on Sephadex G-25. The enzyme (20 units/l.) was incubated with hypoxanthine (5×10^{-5}M) until uric acid formation was complete and then the enzyme system was subjected to chromatography on Sephadex G-25. The entire procedure (activation of the enzyme system by incubation with hypoxanthine and chromatography) required approximately 10 min. When epinephrine (1×10^{-4}M) was added to the eluted enzyme, no adrenochrome formation was detectable over a 10-min period; however, when epinephrine and hypoxanthine (5×10^{-5}M) were added to the eluted enzyme, adrenochrome formation proceeded rapidly. The gel filtration studies may indicate therefore that a substance (or substances) of relatively low molecular weight is involved in the xanthine oxidase-mediated oxidation of epinephrine and that the stability of the activated X.O. system is not due simply to, for example, the production of a stable conformational isomer of xanthine oxidase.

A number of hydroxypteridines have been found to be excellent substrates for X.O.[8] and we have found recently[9] that several aminopteridines are oxidized efficiently by this enzyme. We found that oxidation of several pteridine derivatives by X.O. was also capable of inducing the oxidation of epinephrine to adrenochrome (Table 4). There was a reasonably good correlation between the rates of oxidation of the various substrates by X.O. and the rate of formation of adrenochrome from epinephrine in the presence of the substrate. 2-Amino-4-methylpteridine and 4-amino-6,7-dimethylpteridine, which were not oxidized to any appreciable extent by X.O.[9] under the conditions employed, did not induce oxidation of epinephrine.

In experiments with different batches of commercial X.O. prepared by the method of Gilbert and Bergel,[13] considerable variation in epinephrine-oxidizing capacity was noted with the standard assay system as well as with the activated system. It is worth pointing out in this regard that variations[14-17] in the activity of X.O. preparations have been described previously and may be related to variations in flavin coenzyme or metal ion content, or to the presence of an endogenous inhibitor in certain preparations. Variations in aspects of the enzymatic activity of X.O. also may be a result of

TABLE 4. OXIDATION OF EPINEPHRINE IN THE PRESENCE OF SELECTED PTERIDINES*

Pteridine	Reference no.	Rate of oxidation of pteridine†	Rate of oxidation of epinephrine‡
4-Hydroxy	10	80	27
4-Amino	10	3	1
2-Amino	10	30	10
4-Amino-6,7-dimethyl	11	0	0
2-Amino-4-methyl	12	0	0

* All studies were carried out with X.O. (20 units/l.), pteridine substrate (5×10^{-5} M) and epinephrine (1×10^{-4} M) at pH 7·4 and 37°.

† Relative rate; rate of oxidation of hypoxanthine by X.O. taken as 100.

‡ Expressed as μmoles epinephrine oxidized per liter per minute.

alterations[18] produced by proteolytic digestion procedures used routinely in standard procedures for the isolation of the enzyme from milk. We have found that X.O. isolated by the method of Ball,[2] whether obtained commercially or prepared in this laboratory, showed more consistent activity in inducing epinephrine oxidation than did more highly purified preparations obtained by the method of Gilbert and Bergel.[13] It should be noted in this connection that the oxidation of epinephrine by X.O. preparations may require the presence of a component (or components) other than X.O. *per se*, which may be present in different concentrations in different enzymic preparations.

DISCUSSION

The data presented in this communication indicate that epinephrine can be oxidized to adrenochrome *in vitro* through the intermediacy of X.O.; this oxidation will not occur over the time interval studied unless the enzyme first oxidizes a more conventional substrate (such as a purine or a pteridine).

The oxidation of epinephrine in the X.O. system may be initiated by free radicals; radical species have been detected during the course of oxidation of various substrates by X.O.[19–22] Such free radicals have been implicated in the reduction of cytochrome c by X.O.[21] Mazur *et al.*[23] have reported that the oxidation of epinephrine to adrenochrome in a chemical system may be brought about by the action of superoxide radicals. McCord and Fridovich[21] have described recently a scheme for generation of superoxide free radicals when reduced X.O. reacts with molecular oxygen. Prompted by our preliminary report[24] that epinephrine oxidation can occur in the X.O. system, McCord and Fridovich[25] directed their attention to the possible role of superoxide radicals in this process; these workers concluded, based on observations that superoxide dismutase is a potent inhibitor of the X.O.-mediated oxidation of epinephrine, that superoxide radicals are involved in this reaction. However, it must be emphasized that McCord and Fridovich[25] studied epinephrine oxidation in the X.O. system at pH 10·1, and the large pH difference between their reaction system and that used by us may make it difficult to extrapolate their results to our system. Of possible significance in this regard is the observation that the superoxide radical has a half-life of less than 1 sec at pH 10 and appears to be even more unstable at neutral pH.[20] It is worthy of mention that adrenochrome can be produced from epinephrine by cytochrome oxidase

in the presence of cytochrome c,[26] and it has been demonstrated[22] that cytochrome oxidase, like X.O., can induce the formation of free radical species when the appropriate substrate is oxidized.

It is possible that metal ion catalysis is important in the conversion of epinephrine to adrenochrome by X.O. A number of metalloproteins are known to be capable of oxidizing epinephrine and other catecholamines,[27] and metal ions have been found capable of inducing epinephrine oxidation in nonenzymatic systems.[28] Iron in coordinate linkages[28,29] can efficiently oxidize epinephrine. It is conceivable that alteration in the coordination state of a metal ion bound to X.O. (possibly resulting from a conformational change occurring during formation of an enzyme–substrate complex) may be a factor in determining the ability of the enzyme to oxidize epinephrine.

The observation that epinephrine does not stimulate X.O. but rather is itself oxidized in the X.O. system emphasizes the hazards of using optical density changes at a fixed wavelength as the sole spectroscopic index of enzymatic activity. For spectrophotometric studies, it is important that, initially at least, full scans of the absorption spectrum of reaction mixtures be performed in order to validate further kinetic studies at fixed wavelengths.

Adrenochrome formation by the X.O. system *in vitro* may suggest the operation of a similar system *in vivo*. It should be stressed, however, that the major metabolic products derived from epinephrine in man do not include adrenochrome-like substances.[30] Adrenochrome has been reported to produce a wide variety of biochemical and pharmacological effects.[31] Studies *in vitro* have indicated that adrenochrome is capable of inhibiting corticosterone synthesis,[32] and that products derived from adrenochrome can inhibit polypeptide synthesis.[33] The possible role of adrenochrome in the etiology of schizophrenia has been a subject of much controversy over recent years.[34] Adrenochrome formation by brain homogenates has been reported by Vander Wende and Spoerlein,[35] and oxidation of epinephrine and related catecholamines is demonstrable in a variety of other mammalian tissues.[32,36–38] Tyrosinase is also capable of oxidizing catecholamines.[39] Whether adrenochrome formation by any of these systems is of physiological significance remains to be established.

Administration of epinephrine has been found to be associated with development of hyperuricemia in certain patients.[40] Our data *in vitro* might suggest that this hyperuricemic effect of epinephrine is not due to simple stimulation of X.O. activity by epinephrine.

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