A kinetic study of hypoxanthine oxidation by milk xanthine oxidase

Josefa ESCRIBANO, Francisco GARCIA-CANOVAS and Francisco GARCIA-CARMONA*
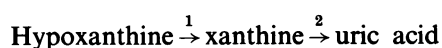Departamento de Bioquimica, Universidad de Murcia, Espinardo, 30001 Murcia, Spain

The course of the reaction sequence hypoxanthine → xanthine → uric acid catalysed by xanthine:oxygen oxidoreductase from milk was investigated on the basis of u.v. spectra taken during the course of hypoxanthine and xanthine oxidations. It was found that xanthine accumulated in the reaction mixture when hypoxanthine was used as a substrate. The time course of the concentrations of hypoxanthine, xanthine intermediate and uric acid product was simulated numerically. The mathematical model takes into account the competition of substrate, intermediate and product and the accumulation of the intermediate at the enzyme. This type of analysis permits the kinetic parameters of the enzyme for hypoxanthine and xanthine to be obtained.

INTRODUCTION

A complex kinetic situation occurs when it is not possible to separate experimentally several coupled reactions, such as the chemical reaction of stabilization of the last product of an enzymic reaction. In previous papers, kinetic analyses of a system of two chemical reactions coupled to an enzymic reaction (Escribano et al., 1984), of one second-order chemical reaction coupled to an enzymic step (Escribano et al., 1985) and of the most complex scheme of dopa (3,4-dihydroxyphenylalanine) oxidation by tyrosinase to dopachrome (Garcia-Carmona et al., 1982) have been carried out.

In the present work, the study of two consecutive reactions catalysed by the same enzyme has been approached, using the bifunctional enzyme milk xanthine oxidase (xanthine:oxygen oxidoreductase, EC 1.2.3.2) that catalyses the two reactions in purine catabolism in mammals:

$$\text{Hypoxanthine} \xrightarrow{1} \text{xanthine} \xrightarrow{2} \text{uric acid}$$

Owing to the fact that both reactions are sequential, kinetic study of the hypoxanthine oxidation reaction is very difficult, because the second activity upon xanthine (step 2) occurs simultaneously with the formation of the xanthine as the product of the first one (step 1). Because of this, most kinetic studies of xanthine oxidase have been limited to its second activity (Gutfreund & Sturtevant, 1959; Ackerman & Brill, 1962; Jezewska, 1973; Olson et al., 1974).
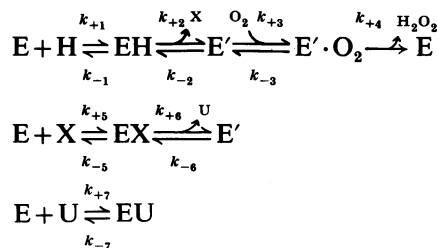
The attempts made in relation to the analysis of the global activity of xanthine oxidase with hypoxanthine as substrate have led to some very general ideas about this process, such as that the xanthine/uric acid ratio depends on three factors: (a) time of reaction, (b) enzyme concentration and (c) hypoxanthine concentration (Jezewska, 1973). However, owing to the procedure used, it is not possible to make a correct determination of the Michaelis constants of xanthine oxidase (Jezewska, 1973) or the related enzyme xanthine:NAD^+ oxidoreductase (Kaminski & Jezewska, 1981, 1985) for hypoxanthine.

The present paper describes kinetic experiments for studying the time course of the overall reaction and computer simulations to determine the kinetic parameters of each reaction catalysed by xanthine oxidase. Likewise a new spectrophotometric method that permitted a continuous evaluation of hypoxanthine, xanthine and uric acid concentrations in the reaction medium was set up.

THEORY

Xanthine oxidase and xanthine dehydrogenase catalyse the oxidation of xanthine by oxidizing substrate such as O_2 and NAD^+ respectively. The effects of varying the concentration of the reducing substrate at fixed concentrations of the oxidizing substrate, and vice versa, on the activities of bovine milk xanthine oxidase (Fridovich, 1964; Massey et al., 1969), chicken liver xanthine dehydrogenase (Rajagopalan & Handler, 1967) and turkey liver xanthine dehydrogenase (Cleere & Coughlan, 1975) have been reported. In each case double-reciprocal plots of the data gave parallel lines and therefore a ping-pong mechanism has been proposed.

Assuming a ping-pong mechanism for the oxidation of hypoxanthine and xanthine by milk xanthine oxidase and that uric acid could bind to the oxidized or the reduced form of the enzyme owing to the considerable structural similarity to both substrates, the inhibition will be more complex than that of a simple reaction product, and therefore the kinetic scheme will be:

$$E+H \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EH \underset{k_{-2}}{\overset{k_{+2}X}{\rightleftharpoons}} E' \underset{k_{-3}}{\overset{O_2\ k_{+3}}{\rightleftharpoons}} E'\cdot O_2 \overset{k_{+4}\ H_2O_2}{\longrightarrow} E$$

$$E+X \underset{k_{-5}}{\overset{k_{+5}}{\rightleftharpoons}} EX \underset{k_{-6}}{\overset{k_{+6}\ U}{\rightleftharpoons}} E'$$

$$E+U \underset{k_{-7}}{\overset{k_{+7}}{\rightleftharpoons}} EU$$

where E is the oxidized enzyme, E' is the reduced enzyme, H is hypoxanthine, X is xanthine and U is uric acid.

* To whom correspondence should be addressed.

Assuming the steady-state conditions and considering that the study is performed under saturating concentration of O_2 (0.26 mM) (Ackerman & Brill, 1962; Fridovich, 1964; Massey *et al.*, 1969), which means kinetically that $[O_2] \to \infty$, the following equations for the variations of hypoxanthine, xanthine and uric acid concentrations with time can be obtained:

$$\frac{-d[H]}{dt} = \frac{V_{max.}^{H}[H]}{K_m^H + [H] + \frac{K_m^H[X]}{K_m^X} + \frac{K_m^H[U]}{K_i}} \quad (1)$$

$$\frac{d[X]}{dt} = \frac{V_{max.}^{H}[H]K_m^X - V_{max.}^{X}[X]K_m^H}{K_m^H K_m^X + K_m^X[H] + K_m^H[X] + \frac{K_m^H K_m^X[U]}{K_i}} \quad (2)$$

$$\frac{d[U]}{dt} = \frac{V_{max.}^{X}[X]}{K_m^X + \frac{K_m^X[H]}{K_m^H} + [X] + \frac{K_m^X[U]}{K_i}} \quad (3)$$

Eqns. (1)–(3) show the rates of variation of substrate, intermediate and product concentrations in terms of the kinetic parameters of the system, such as K_m^H, K_m^X, K_i, $V_{max.}^H$ and $V_{max.}^X$, and so it would be possible to evaluate these from the [hypoxanthine]–time, [xanthine]–time and uric acid–time profiles.

Data analysis and simulation

Computations were performed using a program written in BASIC and run on an Olivetti M-20 computer. The absorbance data of the kinetic assays were fitted by non-linear regression coupled to the numeric integration of the differential eqns. (1), (2) and (3).

The corresponding kinetic parameters, $V_{max.}$ and K_m, to hypoxanthine and xanthine were determined by using non-linear regression by the Marquardt algorithm (Marquardt, 1963), whereas for the numeric integration a method of numerical integration of fourth order of Runge–Kutta (Bronson, 1981) was used.

MATERIALS AND METHODS

Materials

Milk xanthine oxidase was purchased from Sigma Chemical Co. and had a specific activity of 1.1 units/mg of protein. Hypoxanthine, xanthine and uric acid also came from Sigma Chemical Co. One unit of enzyme is defined as the amount that transforms 1.0 μmol of xanthine into uric acid per min at pH 8.0 at 25 °C.

All solutions were prepared in 30 mM-Tris/HCl buffer, pH 8.0.

Methods

The u.v. standard spectra of hypoxanthine, xanthine and uric acid (Fig. 1) were obtained on a Perkin–Elmer Lambda-3 spectrophotometer on-line-interfaced with a Perkin–Elmer computer model 3600 data-station, with 30 mM-Tris/HCl buffer, pH 8.0, at 25 °C.

The difference spectra of the enzyme reaction mixtures were obtained with the reference buffer containing an amount of enzyme solution equal to that in the reaction mixtures.

The spectrophotometric measurements of enzymic activity were carried out with the aid of an Aminco DW-2 spectrophotometer, equipped with an Aminco–
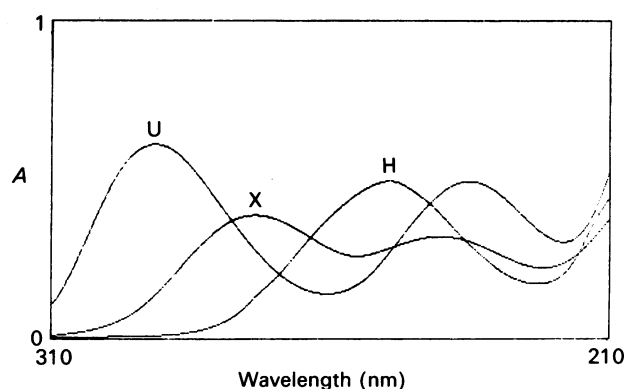

Fig. 1. U.v. spectra of hypoxanthine (H), xanthine (X) and uric acid (U)

Hypoxanthine, xanthine and uric acid were at equimolar concentrations (60 μM) in 30 mM-Tris/HCl buffer, pH 8.0, at 25 °C. Scan speed was up to 60 nm/min.

Morrow stopped-flow accessory and a Dasar T.M. unit in order to improve the reproducibility of the measurements.

Xanthine oxidase activity was assayed by monitoring the hypoxanthine disappearance at 279.9 nm, the isosbestic point of xanthine and uric acid. Xanthine formation and its disappearance were spectrophotometrically determined at 271.0 nm, the isosbestic point of hypoxanthine and uric acid, and uric acid accumulation was monitored at 264.6 nm, the isosbestic point of hypoxanthine and uric acid.

The concentrations of hypoxanthine, xanthine and uric acid at each time were evaluated according to the following expressions:

$$[H]_{279.9}^{t} = (A_{279.9}^{0} - A_{279.9}^{t})/\epsilon_{279.9}^{H} \quad (4)$$

$$[X]_{271.0}^{t} = (A_{271.0}^{t} - A_{271.0}^{0})/\epsilon_{271.0}^{X} \quad (5)$$

$$[U]_{264.6}^{t} = [H]^{0} - (A_{264.6}^{t} - A_{264.6}^{0})/\epsilon_{264.6}^{U} \quad (6)$$

where the subscript indicates the wavelength and the superscript the reaction time.

For this purpose the apparent molar absorption coefficients were determined: $\epsilon_{279.9}^{H} = 6.13 \times 10^3$, $\epsilon_{271.0}^{X} = 4.08 \times 10^3$ and $\epsilon_{264.6}^{U} = 3.85 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

RESULTS AND DISCUSSION

By using spectroscopic scanning techniques the reaction intermediates can be detected. This technique was used to measure xanthine as a transient in the hypoxanthine → uric acid reaction. The spectral pattern obtained during the hypoxanthine oxidation by xanthine oxidase is shown in Fig. 2(*a*), in which no isosbestic point appears. With time there was a disappearance of the maximum at 249 nm corresponding to hypoxanthine and an initial increase of absorption at 270 nm corresponding to the increase of the xanthine concentration in the reaction medium. In addition, there was observed the appearance of two maxima at 293 nm and 238 nm due to the uric acid formation.

The graphic analysis application procedure of Coleman *et al.* (1970) to this spectral set showed a good fit related to the existence of three different species kinetically correlated (Fig. 2*b*), these being obviously hypoxanthine, xanthine and uric acid, since during the
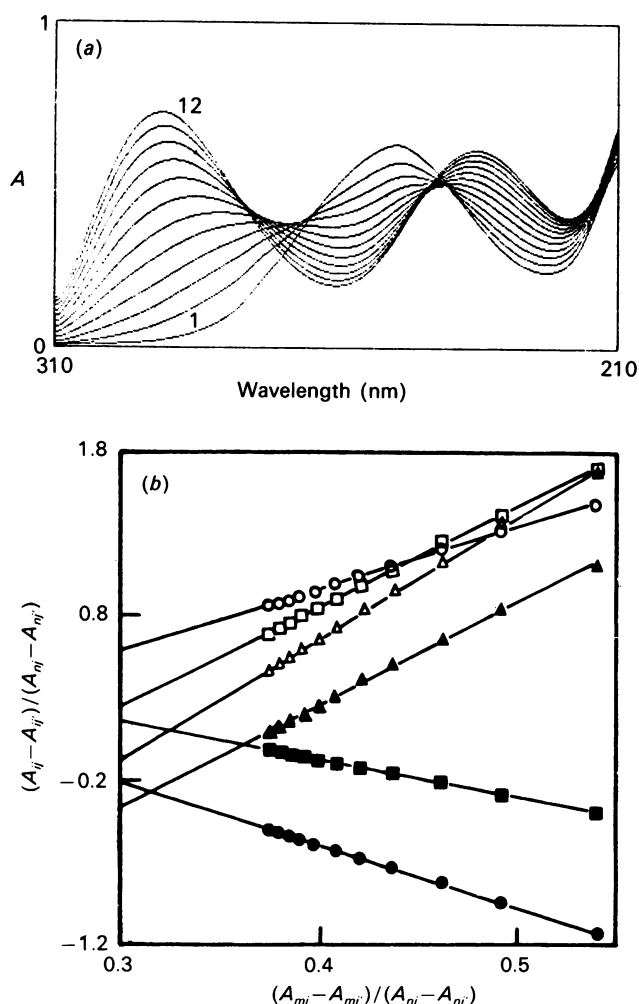
Fig. 3. Spectrophotometric recordings for the oxidation of xanthine by xanthine oxidase

Xanthine (60 μM) was oxidized by xanthine oxidase (3.1 μg/ml) at 25 °C in 30 mM-Tris/HCl buffer, pH 8.0. Scan speed was up to 60 nm/min at 40 s intervals.

Fig. 2. Hypoxanthine oxidation by xanthine oxidase

(a) Spectrophotometric recordings (from 210 to 310 nm) for the oxidation of hypoxanthine (60 μM) by xanthine oxidase (3.1 μg/ml) at 25 °C in 30 mM-Tris/HCl buffer, pH 8.0, are shown. Scan speed was up to 60 nm/min at 40 s intervals. (b) Graphic analysis of spectra recorded in (a). In this analysis A_{ij} is the absorbance at wavelength i obtained during tracing j, so that A_{21} is the absorbance at 253 nm obtained during the first tracing of the absorption spectrum. Selected wavelengths were: 242 nm (■), 253 nm (●), 271 nm (▲), 277 nm (△), 281 nm (□), and 285 nm (○); $m = 227$ nm; $n = 291$ nm; $j' =$ tracing 1. The test for three species with restrictions was applied.

hypoxanthine oxidation by xanthine oxidase the intermediate accumulation of xanthine and formation of uric acid as final product are produced.

When the study of the second reaction catalysed by xanthine oxidase was carried out, the disappearance of two maxima corresponding to the xanthine spectrum was shown (Fig. 3), accompanied by the appearance of the two characteristic maxima of uric acid and two isosbestic points (279.9 and 246.4 nm), which were identical with those shown in Fig. 1. The application of the graphic analysis procedure of Coleman et al. (1970) to the experimental traces gave a good fit related to the existence of two different kinetically related species absorbing in solution (results not shown).
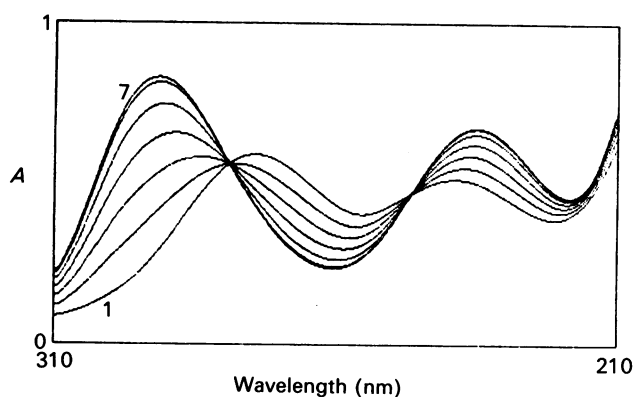
Determination of the kinetic constants of xanthine oxidase for xanthine

As an initial step in the study of the overall reaction of xanthine oxidase, the kinetic constants for the individual reaction of xanthine oxidation were determined by means of steady-state velocity studies of the xanthine → uric acid reaction with different substrate concentrations.

The spectrophotometric measurements were carried out at 293.0 nm, corresponding to the absorbance maximum of the uric acid spectrum ($\epsilon = 10$ mM$^{-1} \cdot$cm^{-1}) (Heinz et al., 1979).

The K_m for xanthine obtained (3.7 μM) was similar to that obtained by other authors (Jezewska, 1973; Olson et al., 1974; Morpeth, 1983).

Steady-state-velocity studies with a xanthine concentration range from 11.5 to 47.1 μM and in the presence of different uric acid concentrations from 11.7 to 161.5 μM showed in Dixon plots that uric acid acts as a linear competitive inhibitor ($K_i = 178$ μM) (results not shown). This value was introduced as a constant in the non-linear-regression analysis, since its high value indicates a limited contribution of this step.

Kinetics of the overall reaction

The oxidation of xanthine to uric acid catalysed by xanthine oxidase has been extensively studied by several authors (Jezewska, 1973; Olson et al., 1974; Morpeth, 1983), with evaluation of the corresponding kinetic parameters. However, the step of xanthine accumulation from hypoxanthine oxidation has been hardly approached from the kinetic point of view, since it has not been possible to determine the kinetic parameters of that reaction, either from the xanthine accumulation, which is the substrate of the second reaction, or from the quantification of the final product of the two consecutive reactions catalysed by xanthine oxidase, i.e. uric acid.

This problem was approached by Jezewska (1973), who tried to determine the K_m for hypoxanthine by measuring the rate of accumulation of uric acid at 290 nm; she obtained only an apparent linearity from Lineweaver–Burk plots with a very low hypoxanthine concentration, concluding that the K_m^H value changes with the time course of the reaction. Obviously, this does not seem probable, since a dynamic fitting between the
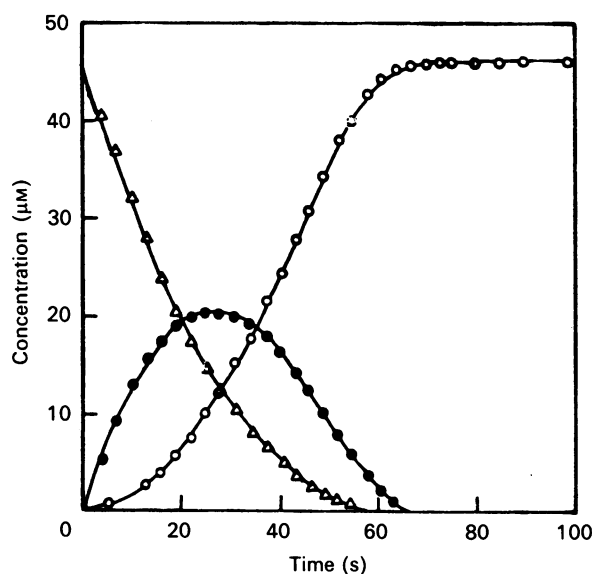
Fig. 4. Comparison of experimental data for the time course of the overall reaction with simulations

The points represent the experimental data obtained from a reaction mixture that contained 47 μM-hypoxanthine and xanthine oxidase (0.27 mg/ml) in 30 mM-Tris/HCl buffer, pH 8.0. $\triangle$, Hypoxanthine; $\bullet$, xanthine; $\bigcirc$, uric acid. The lines are computer simulations using the kinetic parameters: $V_{max.}^H = 1.69\ \mu$M·s^{-1}, $K_m^H = 1.86\ \mu$M, $V_{max.}^X = 2.07\ \mu$M·s^{-1}, $K_m^X = 3.38\ \mu$M and $K_i = 0.178$ mM.

hypoxanthine, xanthine and uric acid concentrations with time is likely to occur, modifying the overall kinetics of the system. Therefore, in order to evaluate the kinetic parameters of the system and mainly $V_{max.}$ and K_m for hypoxanthine, studies of the developing changes in the overall system with time have to be carried out.

The kinetics of the overall reaction were studied by incubating the enzyme with hypoxanthine, in the absence or in the presence of xanthine or uric acid, and monitoring the disappearance of hypoxanthine (at 279.9 nm), the appearance and oxidation of xanthine (at 271.0 nm) and the accumulation of uric acid (at 264.6 nm) during the reaction (Fig. 4).

The kinetic parameters $V_{max.}$ and K_m for hypoxanthine and xanthine were evaluated by non-linear regression by fitting of four parameters, by using initial estimations of K_m and $V_{max.}$ values for xanthine experimentally determined. The K_m and $V_{max.}$ values assigned for hypoxanthine were similar to those for xanthine, since experimentally it was proven that the uptake of half concentration of hypoxanthine coincided with the maximum xanthine accumulation.

The fitting by non-linear regression was carried out for three different experimental situations of hypoxanthine oxidation by xanthine oxidase: (a) hypoxanthine oxidation; (b) hypoxanthine oxidation in the presence of xanthine; (c) hypoxanthine oxidation in the presence of uric acid.

The kinetic parameters corresponding to the overall reaction obtained under these experimental situations were very similar, and hence the weighted mean of each one was evaluated, and is shown in Table 1.

When the theoretical curves obtained by simulation of eqns. (1), (2) and (3) by using the kinetic parameters

Table 1. Apparent Michaelis constants and maximum velocities for substrates of xanthine oxidase

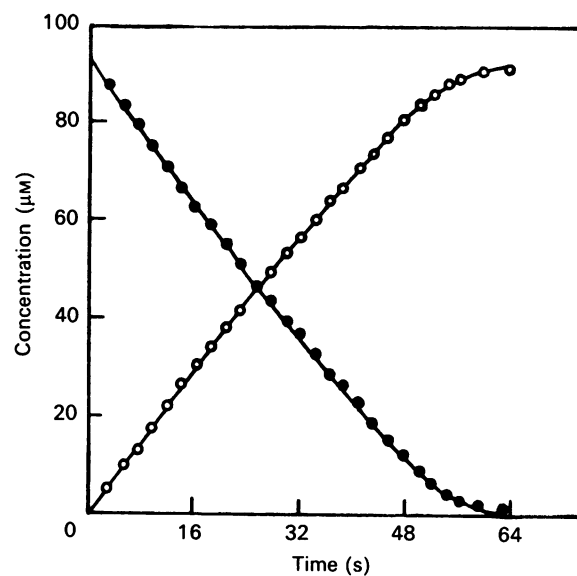| Substrate	K_m (μM)	$V_{max.}$ (μM·s^{-1})
Hypoxanthine	1.86 ± 0.1	1.69 ± 0.07
Xanthine	3.38 ± 0.17	2.07 ± 0.02

Fig. 5. Xanthine oxidation by xanthine oxidase

The points represent the values obtained experimentally from a reaction medium that contained 93 μM-xanthine and xanthine oxidase (0.27 mg/ml) in 30 mM-Tris/HCl buffer, pH 8.0, at 25 °C. $\bullet$, Xanthine; $\bigcirc$, uric acid. The lines are the results of the simulation using the kinetic parameters: $V_{max.}^X = 1.91\ \mu$M·s^{-1} and $K_m^X = 3.38\ \mu$M, obtained from the adjustment by non-linear regression. $K_i = 0.178$ mM.

calculated by non-linear regression were superimposed on the data experimentally obtained (Fig. 4), a good agreement between the predicted behaviour and the experimental results was obtained.

In order to complete the study and to confirm the kinetic parameters obtained for the overall reaction, analysis of the reaction of xanthine oxidation by xanthine oxidase in the absence of hypoxanthine was carried out.

By using as initial values those obtained by non-linear regression analysis of the overall reaction, the fitting by non-linear regression of the respective parameters study of the progress curves led to K_m and $V_{max.}$ values for xanthine that showed a high concordance with regard to the initial estimations. At the same time, the superposition of the theoretical curves simulated from the kinetic parameters obtained by non-linear regression with the curves obtained experimentally (Fig. 5) was good enough. The values of those parameters coincided with the ones determined by the analysis of initial rates at different xanthine concentrations.

The results obtained in the present work are in

agreement with a ping-pong mechanism for the xanthine oxidase catalysis in its activity upon xanthine as has been extensively proposed (Fridovich, 1964; Massey *et al.*, 1969; Morpeth, 1983) as well as in its activity upon hypoxanthine, with K_m^H $1.72 \pm 0.10\,\mu$M.

The uric acid in the reaction medium acts as a competitive inhibitor even at saturating concentration of O_2, but, however, this is in disagreement with the typical expression for the inhibition by product of the ping-pong mechanism (Roberts, 1977). This can be explained by its close structural similarity to the substrates and therefore it tends to react with the oxidized enzyme form, which permits uric acid to express characteristics of a competitive inhibitor.

REFERENCES

Ackerman, E. & Brill, A. S. (1962) Biochim. Biophys. Acta **56**, 397–412

Bronson, R. (1981) Ecuaciones Diferenciales Modernas, pp. 263–278, McGraw-Hill, Mexico

Cleere, W. F. & Coughlan, M. P. (1975) Comp. Biochem. Physiol. B. **50**, 311–322

Coleman, J. S., Varga, L. P. & Mastin, S. H. (1970) Inorg. Chem. **9**, 1015–1020

Escribano, J., Garcia-Carmona, F., Garcia-Canovas, F., Iborra, J. L. & Lozano, J. A. (1984) Biochem. J. **223**, 633–638

Escribano, J., Garcia-Canovas, F., Garcia-Carmona, F. & Lozano, J. A. (1985) Biochim. Biophys. Acta **831**, 313–320

Fridovich, I. (1964) J. Biol. Chem. **239**, 3519–3521

Garcia-Carmona, F., Garcia-Canovas, F., Iborra, J. L. & Lozano, J. A. (1982) Biochim. Biophys. Acta **717**, 124–131

Gutfreund, H. & Sturtevant, J. M. (1959) Biochem. J. **73**, 1–6

Heinz, F., Reckel, S. & Kalden, J. R. (1979) Enzymes **24**, 239–246

Jezewska, M. M. (1973) Eur. J. Biochem. **36**, 385–390

Kaminski, Z. W. & Jezewska, M. M. (1981) Biochem. J. **200**, 597–603

Kaminski, Z. W. & Jezewska, M. M. (1985) Comp. Biochem. Physiol. B **80**, 371–375

Marquardt, D. W. (1963) J. Soc. Ind. Appl. Math. **11**, 431–441

Massey, V., Brumby, P. E., Komai, H. & Palmer, G. (1969) J. Biol. Chem. **244**, 1682–1691

Morpeth, F. F. (1983) Biochim. Biophys. Acta **744**, 328–334

Olson, J. S., Ballou, D. P., Palmer, G. & Massey, V. (1974) J. Biol. Chem. **249**, 4350–4362

Rajagopalan, K. V. & Handler, P. (1967) J. Biol. Chem. **242**, 4097–4107

Roberts, D. V. (1977) Enzyme Kinetics, p. 133, Cambridge University Press, Cambridge