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Xanthine Oxidase Catalyzes the Synthesis of Retinoic Acid*

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Milk xanthine oxidase (xanthine: oxygen oxidoreductase; XO; EC 1.1.3.22) was found to catalyze the conversion of retinaldehyde to retinoic acid. The ability of XO to synthesize all *trans*-retinoic acid efficiently was assessed by its turnover number of 31.56 min^{-1}, determined at pH 7.0 with 1 nM XO and all *trans*-retinaldehyde varying between 0.05 to 2 μM. The determination of both retinoid and purine content in milk was also considered in order to correlate their concentrations with kinetic parameters of retinaldehyde oxidase activity. The velocity of the reaction was dependent on the isomeric form of the substrate, the all *trans*- and 9-*cis*-forms being the preferred substrates rather than 13-*cis*-retinaldehyde. The enzyme was able to oxidize retinaldehyde in the presence of oxygen with NAD or without NAD addition. In this latter condition the catalytic efficiency of the enzyme was higher. The synthesis of retinoic acid was inhibited 87% and 54% by 4 μM and 2 μM allopurinol respectively and inhibited 48% by 10 μM xanthine in enzyme assays performed at 2 μM all *trans*-retinaldehyde. The K_i value determined for xanthine as an inhibitor of retinaldehyde oxidase activity was 4 μM.

Keywords: Retinoic acid, Xanthine oxidase, Retinaldehyde oxidase, Purine inhibition

Abbreviations: ADH, aldehyde dehydrogenase; HPLC, high performance liquid chromatography; RAR, retinoic acid receptor; RXR, retinoid X receptor; Tris, tris(hydroxymethyl)aminomethane; XDH, xanthine dehydrogenase; XO, xanthine oxidase

INTRODUCTION

The observation that milk xanthine oxidase [XO], an enzyme of broad specificity,[1,2] is capable of converting retinaldehyde to retinoic acid[3–5] has not been taken into due consideration. Many data have been reported on the existence of retinaldehyde dehydrogenase or oxidases,[3,5–16] and although specific potential functions in retinoic acid biosynthesis and metabolism can be assigned to several of these enzymes,[17,18] only some of them have been purified and characterized.[19–22]

The well-known importance of the various biological roles and effects of retinoic acid, such as the control of cell proliferation, differentiation

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and morphogenesis, epithelium protection and prevention effects in carcinogenesis,[23–27] stimulated us to attempt to define the conditions in which XO can best act and to determine its catalytic efficiency versus retinaldehyde in various experimental assay conditions. The effect of purines on enzyme activity was also considered.

EXPERIMENTAL

Materials

Dialysis tubing, FAD, NAD, phosphate and pyrophosphate salts, 9-*cis*, 13-*cis*, and all *trans*-retinaldehyde, 13-*cis* and all *trans*-retinoic acid, all *trans*-retinol and all *trans*-retinyl palmitate, trizma pre-set crystals, milk xanthine oxidase, hypoxanthine, xanthine, uric acid and allopurinol (4-hydroxypyrazolo [3,4-d] pyrimidine) were purchased from Sigma Chemical Co. Ammonium acetate, antimony chloride, benzene, chloroform, hydroxylamine, salt for buffers, organic solvents for chromatography and silica gel plates were purchased from Merck. Acetonitrile, dioxane, hexane, methanol and petroleum ether were from Carlo Erba (Italy). Nitrogen-oxygen mixtures were supplied by Alphagaz (France). Centripep-10 filters were from Amicon. μBondapak C_{18}, Nova-Pack C_{18}, μPorasil and Shodex Protein-Pak columns were from Waters. Salts for buffers, organic solvents for chromatography and silica gel plates were from Merck.

Enzyme Source

Milk XO from Sigma (batch X-4500, 1.0–1.3 units/mg protein), diluted with 50 mM Tris-HCl, pH 7.0, was dialyzed with the same buffer for 4–5 h to remove ammonium sulfate and sodium salicylate.[28] This preparation, spectrophotometrically analyzed, exhibited an A_{280}/A_{450} ratio of 4.3 and an A_{450}/A_{550} ratio of 3.9. The corresponding values reported in the literature for the enzyme purified from milk are 5–6 and 2.8, respectively.[28,29,30] The mass value used for xanthine oxidase was 300,000 daltons.[30]

A check of enzyme purity by chromatography was performed by analysis of milk XO by gel filtration on a Shodex Protein-Pak SW 300 column (8 × 300 mm), using a Gilson chromatograph (series 4000). The HPLC apparatus consisted of two model 306 pumps, a 811B dynamic mixer, a model 234 auto injector equipped with 20 μl injection loop, and a 116 model variable-wavelength UV detector. Data acquisition and elaboration were performed by a UniPoint (v1. 71) HPLC System Controller Software. In the chromatographic profile shown in Figure 1 the xanthine oxidase peak is preponderant over the other contaminant protein peaks, accounting for about 80% of the total protein evidenced by the chromatography pattern.

Retinaldehyde Solution

Fifty or 100 μl aliquot of retinaldehyde in ethanol (2.84 mg/ml) was suspended in 100 ml of water by vortexing before use.

Enzyme Assays

Retinaldehyde Oxidase Activity Assay

The medium (200 μL) contained 50 mM Tris-HCl, pH 7.0, 2 μM all *trans*-retinaldehyde, and 1 nM XO. For other assays the concentration of FAD was kept at 1 or 100 nM, that of NAD in the range 1–5 μM, and the variation in retinaldehyde concentration was in the range 0.05–2 μM. The assay mixture was equilibrated with nitrogen-oxygen mixtures for 60 min in a Dubnoff metabolic shaking incubator at 37 °C. The enzyme aliquot was added and after incubation for 10 min the reaction was stopped by the addition of 1 mL ethanol, 5 mL petroleum ether and 1.5 mL water. The extract was evaporated under a nitrogen stream and stored at −20 °C before analysis. The enzyme activity was linear with respect to protein concentration in the range

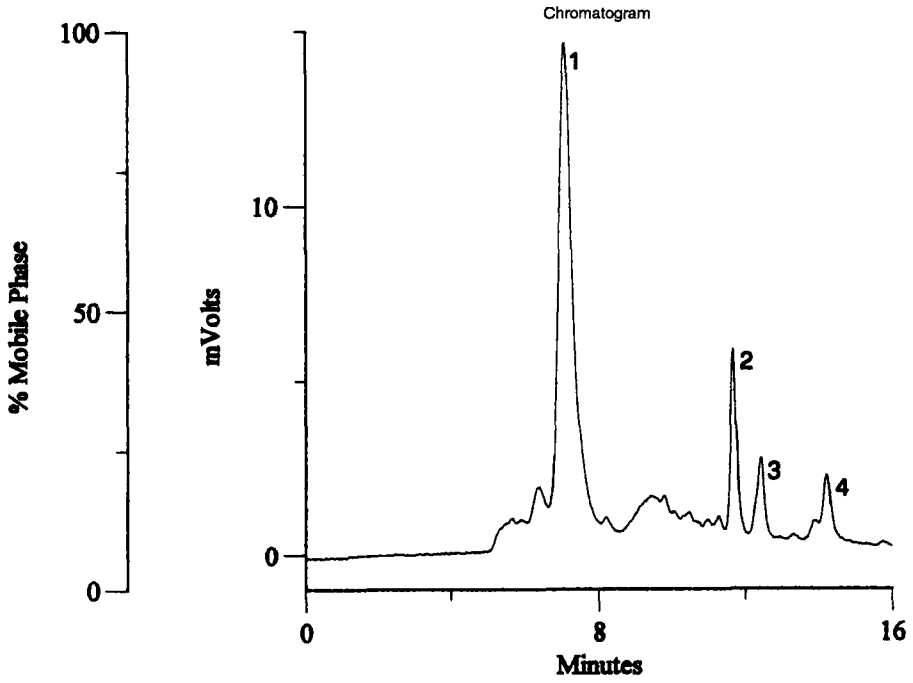

FIGURE 1 Analysis of xanthine oxidase by HPLC gel filtration. 6 μg of milk xanthine oxidase (Sigma X-4500 batch), dialyzed for 5 h in 50 mM Tris-HCl, pH 8.0, was overlaid on a Shodex Protein KW-803 column and eluted at 1 mL min^{-1} with 50 mM Tris-HCl, pH 8.0. Detection was at 280 nm, AUFS 0.01. When desired the eluted xanthine oxidase peak was collected. Peak 1, xanthine oxidase (retention time 7.68), peaks 2, 3 and 4 unidentified contaminants. The pattern reported is the best resulting from three analyses.

0.2–1 nM. The exposure of samples to light was avoided.

XO Activity Assay

One enzyme assay was performed following the conversion of xanthine to uric acid spectrophotometrically at 295 nm at 25 °C using a Beckman DU 70 spectrophotometer according to the procedure of Avis *et al.*[31] reported by Massey *et al.*[29] Alternatively, for the study on the inhibitory effect by retinaldehyde, XO was assayed with a 1 mL medium containing 0.2–1 nM enzyme, 2 to 20 μM xanthine, 50 mM Tris-HCl buffer, pH 7.0 and 1, 2 or 4 μM all *trans*-retinaldehyde.

Separation of xanthine and uric acid was performed following the procedure of Kock *et al.*[32] on a 4 μm Nova-Pak C_{18} column (3.9 × 300 mm) eluting with 50 mM potassium dihydrogen phosphate-phosphoric acid buffer, pH 4.6, at a flow rate of 2 mL min^{-1}. Detection was at 264 nm.

pH Curve Activity

Retinaldehyde oxidase was assayed in the pH range 3.5–9.0 using as buffers 50 mM citrate-phosphate, pH 3.5–7.6, 50 mM Tris-HCl, pH 6.7–8.75 and 50 mM sodium pyrophosphate in the pH range 8.0–9.0, at 1 and 2 μM all *trans*-retinaldehyde, and showed a maximum activity at pH 6.5–7 (Figure 2). In this study for practical reasons Tris-HCl was used as the buffer (Trizma pre-set crystals furnished by Sigma), because of declared pH values relative to temperature.

Retinoid Chromatography

The chromatography apparatus for the analysis of retinoic acid consisted of a Perkin Elmer 410

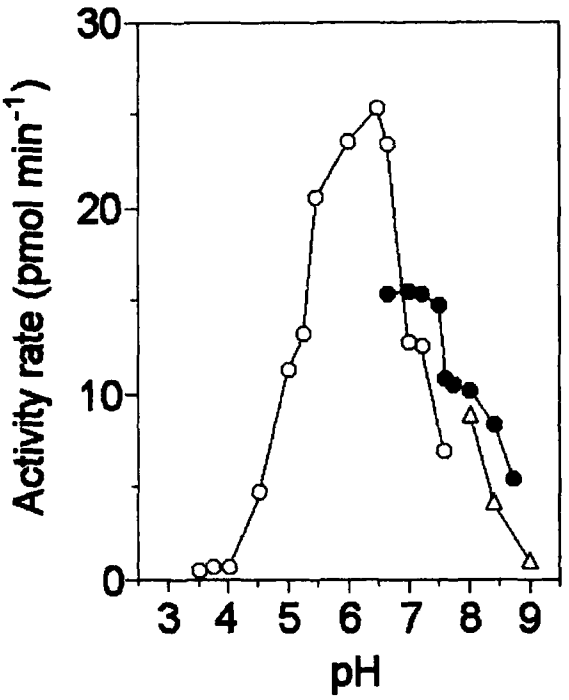

FIGURE 2 pH-dependence of retinaldehyde oxidase activity of xanthine oxidase. The activity was assayed at 37 °C with citrate-phosphate buffer in the pH range 3.5–7.6 (○), with Tris-HCl in the range 6.7–8.75 (●), and with sodium pyrophosphate in the range 8.0–9.0 (△) at 2 μM all *trans*-retinaldehyde.

LC pump, a Rheodyne injector, a Waters μBondapak C_{18} column (3.9 × 300 mm), a Perkin Elmer LC 95 UV/visible spectrophotometer detector and a Perkin Elmer LCI-100 laboratory computing integrator. Acetonitrile-10 mM ammonium acetate (65:35) or methanol – 10 mM ammonium acetate (75:25) were used as eluents.[33,34] Retinoic acid isomers were detected at 345 nm.

Analysis of retinols, retinaldehydes and retinyl esters was performed with a Waters chromatography instrumentation consisting of a 510 pump, a U6K injector, a 10 μm-μPorasil 3.9 × 300 mm column, a 480 LC spectrophotometer and a 730 data integrator. The mobile phase was 7% dioxane in hexane for retinols, 2% dioxane in hexane for retinaldehydes and 0.2% dioxane in hexane for retinyl esters, as previously described.[35] Retinaldehyde oximes were separated and detected using a standard procedure.[36]

Milk Retinoid Content

To determine retinol, retinyl ester and retinoic acid content, 50 mL of fresh bovine milk was extracted with ethanol, light petroleum and water in the ratio 1:5:10:4. Retinaldehyde was extracted as the oxime. Samples with addition of all *trans*-retinol or all *trans*-retinyl palmitate or all *trans*-retinaldehyde oxime or all *trans*-retinoic acid were assayed to determine the extent of retinoid recover.

Retinoid Preparation

Some retinoids were purchased from Sigma and all others not commercially available were prepared in our laboratory as previously reported.[35] 9-*cis*-Retinoic acid was prepared by isomerizing a 0.1% solution of all *trans*-retinoic acid in methanol by light irradiation (300 W lamp) for 30 min at 4 °C and separating the *cis*-form of retinoic acid of the isomerate from the all *trans*-form on silica gel plates, developed with benzene, chloroform and methanol (4:1:1), and stained with $SbCl_3$ in chloroform. The band on the chromatogram (unstained) corresponding to the *cis*-forms of retinoic acid was scraped off and the retinoids were extracted using methanol. This extract was analyzed by HPLC using a series of two μBondapak columns (3.9 × 300 mm) eluted with methanol-10 mM ammonium acetate (75:25) at a flow rate of 0.8 mL min^{-1} (3000 psi) using the chromatographic system described previously. The 9-*cis*-retinoic acid, provisionally identified by excluding the 13-*cis*-retinoic acid and all *trans*-retinoic acid standards, was collected by iterative chromatography separations and its identity confirmed by comparison with a standard of 9-*cis*-retinoic acid given by Hoffman La Roche. Handling of retinoids was always carried out in dim red light.

Milk Purine Content

To determine xanthine and uric acid concentrations, NaOH was added to unpasteurized cow's milk to a final concentration of 0.1 M NaOH. This was centrifuged twice for 30 min at $1500 \times g$ to separate cream from serum. After ultrafiltration on Centripep-10 to discard protein and residual fat, milk serum was saved at $-20\,^{\circ}C$.

Kinetic Data Processing

A provisional estimate of kinetic constants was made with the aid of the Grafit program by Leatherbarrow (Sigma). The appropriate velocity equations describing the kinetic behaviors reported by Segel[37] were verified.

RESULTS

Formation of Retinoic Acid by Milk Xanthine Oxidase as a Function of the Substrate Form

Milk XO incubated with all *trans*-retinaldehyde was able to convert retinaldehyde to retinoic acid (Figure 3 and Table I). The enzyme was less active with 9-*cis*-retinaldehyde and 13-*cis*-retinaldehyde (Figure 4 and Table I), but its catalytic efficiency (K_{cat}/K_m) versus 9-*cis*-retinaldehyde was only three times less than all *trans*-retinaldehyde (Table I).

Influence of Oxygen, NAD or FAD and Catalytic Efficiency of Retinaldehyde Oxidase

In Figure 5, the rate of oxidation of all *trans*-retinaldehyde in the concentration range 0.05–2 μM by XO at the fixed oxygen concentrations of 50, 250, 500 and 750 μM, in the absence of exogenous cofactors such as FAD or NAD is reported. Apparent rate parameters of retinaldehyde oxidase activity depending on different oxygen concentrations are reported in the inset table in Figure 5 together with the kinetic curves

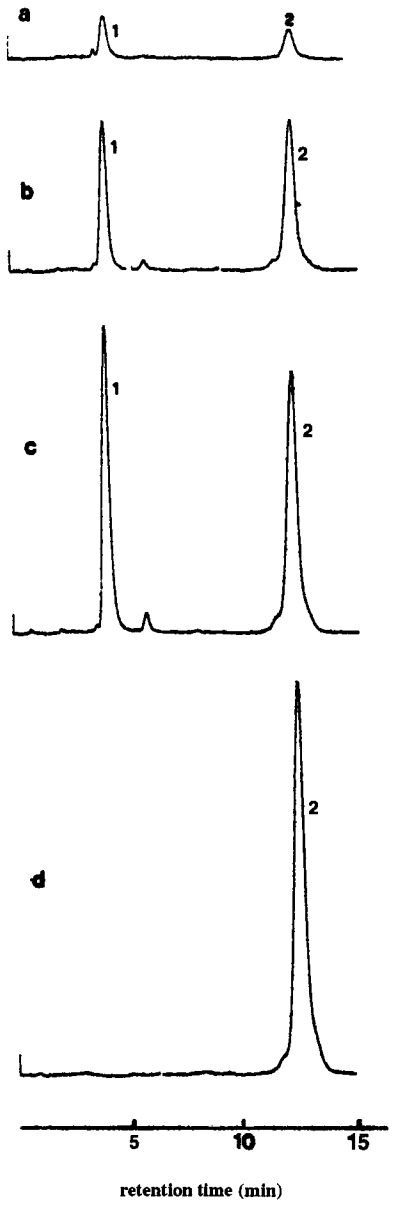

FIGURE 3 Analysis of retinoic acid produced by xanthine oxidase using *trans*-retinaldehyde as the substrate. Dried samples of extracts from xanthine oxidase assays with all *trans*-retinaldehyde (as reported in Materials and Methods) were suspended in acetonitrile-10 mM ammonium acetate (65:35 v/v) and an aliquot (1/5) was analysed by HPLC on a μBondapack C_{18} column (3.9 × 300 mm), eluted with acetonitrile-10 mM ammonium acetate at the same ratio; the flow was 2 mL min^{-1}. Retinoids were monitored at 345 nm. Panels **a**, **b**, and **c**: assays performed with 1 nM enzyme at 0.5, 1, or 2 μM all *trans*-retinaldehyde for 10 min of incubation. Panel **d**: 1 nM enzyme and 2 μM all *trans*-retinaldehyde at time zero. Peaks are as follows: 1, all *trans*-retinoic acid; 2, all *trans*-retinaldehyde.

TABLE I Kinetic parameters for retinaldehyde oxidase activity depending on isomeric substrate form

Substrate	K_{cat} min^{-1}	K_m nM	K_{cat}/K_m† min^{-1} nM^{-1}
trans-retinaldehyde	31.56 ± 2.60	290 ± 25	0.108
9-*cis*-retinaldehyde	9.94 ± 0.82	163 ± 11	0.060
13-*cis*-retinaldehyde	4.09 ● 0.39	187 ± 15	0.021

† Retinaldehyde oxidase activity was assayed in the retinaldehyde concentration range of 0.05–2 μM with 1 nM xanthine oxidase at pH 7.0. Data are mean ± SD of four experiments.

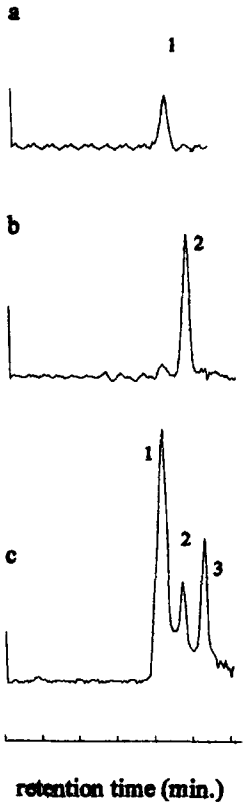

FIGURE 4 Analysis of retinoic acids biosynthesised by xanthine oxidase using 13-*cis*-retinaldehyde or 9-*cis*-retinaldehyde. Dried samples of extract from xanthine oxidase assays with 13-*cis* or 9-*cis*-retinaldehyde were suspended in methanol-10 mM ammonium acetate (75:25 v/v) and an aliquot (1/5) was analyzed on two μBondapak C_{18} columns in series, eluting with methanol-10 mM ammonium acetate (75:25) at a flow rate of 0.8 ml min^{-1}. Detection was at 345 nm. Panel **a**: 10 min reaction of 1 nM xanthine oxidase with 1 μM 13-*cis*-retinaldehyde, at pH 7.0. Panel **b**: 10 min reaction of 1 nM xanthine oxidase with 1 μM 9-*cis*-retinaldehyde. Rate activities and K_m values determined for these reactions are reported in Table I. Panel **c**: retinoic acid standard peaks are as follows: 1, 13-*cis*-retinoic acid; 2, 9-*cis*-retinoic acid; 3, all *trans*-retinoic acid.

from which their values were obtained. The highest catalytic efficiency (K_{cat}/K_m) of retinaldehyde oxidase was shown when this system was incubated at 50 μM oxygen; the increase in oxygen concentration did not improve the system's efficiency (inset table in Figure 5). The addition of 1 or 5 μM NAD to these enzyme assay systems caused an increase of the K_{cat} values and a more significant increase of K_m values. Consequently, a halved catalytic efficiency was observed in the presence of NAD. On the other hand, the addition of 2 or 100 nM FAD to the base enzyme assays caused similar variations of the K_{cat}/K_m ratio (data not shown). Because of these negative effects on XO kinetics exerted by NAD or exogenous FAD, routine assays were performed without them.

Influence of Xanthine and Purine Analogues on Retinaldehyde Oxidase Activity

In order to assess whether and to what extent the concentration of xanthine, well known as one of the usual substrates for the enzyme, may influence retinaldehyde oxidase activity, enzyme assays with the addition of xanthine in the range 1–20 μM were performed (Table II). At concentrations higher than 1 μM an inhibitory effect was observed. The inhibition constant (K_i) for xanthine was 4 ± 0.5 μM in enzyme assays in which the conversion of all *trans*-retinaldehyde to all *trans*-retinoic acid was studied at the fixed concentrations of 2, 4, 10, and 20 μM xanthine (Figure 6). Allopurinol exerted its well-known inhibiting effect (26–87%) in the concentration range 1–4 μM (Table II).

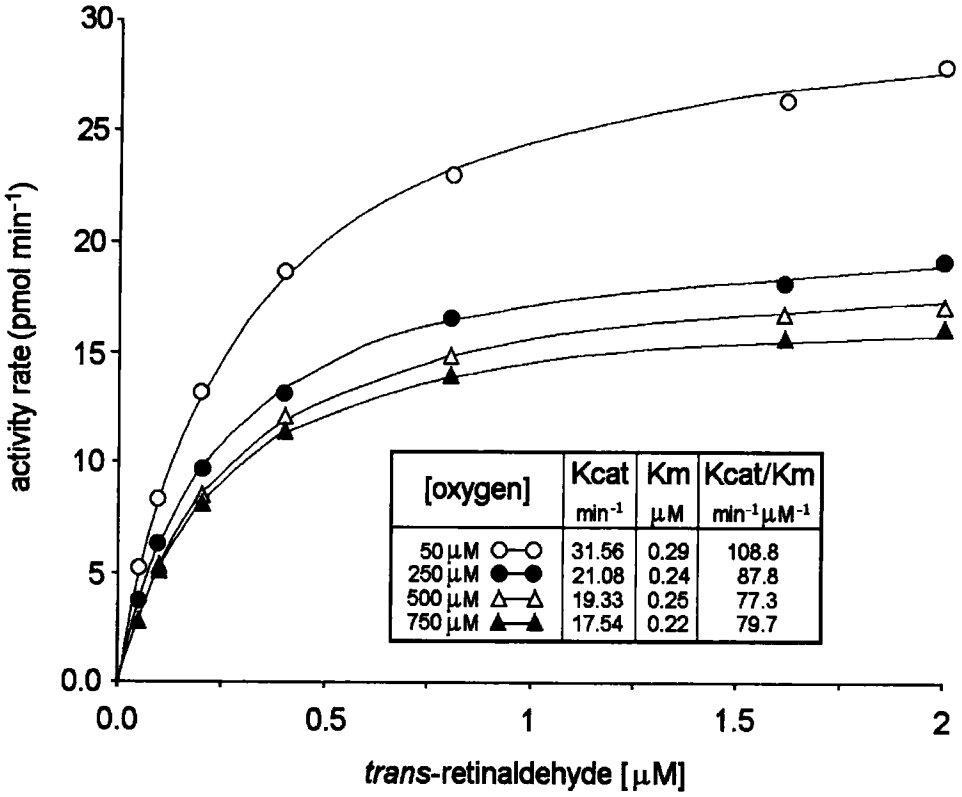

FIGURE 5 Kinetic patterns of retinaldehyde oxidase activity by xanthine oxidase assayed with all *trans*-retinaldehyde at different oxygen concentrations. Activity assayed with oxygen at 50 μM (○), at 250 μM (●), at 500 μM (△), and 750 μM (▲). Rate parameters relative to these curves are shown in the inset table.

TABLE II Effects of xanthine and allopurinol at various concentrations on the retinaldehyde oxidase activity of xanthine oxidase

Effector	Retinaldehyde Oxidase[†] percentage activity
1 μM xanthine	92 ± 7
2 μM xanthine	80 ± 8
4 μM xanthine	68 ± 4
10 μM xanthine	46 ± 3
20 μM xanthine	32 ± 4
1 μM allopurinol	74 ± 5
2 μM allopurinol	46 ± 3
4 μM allopurinol	13 ± 2
10 μM allopurinol	4 ± 1
20 μM allopurinol	ND

[†] 100% activity is 27 pmol min^{-1}. The assay was performed incubating 2 μM *trans*-retinaldehyde under 50 μM oxygen, 1 nM xanthine oxidase, 50 mM Tris-HCl, pH 7.0, and xanthine or allopurinol in the concentrations reported above for 10 min at 37 °C. Data are mean of six different experiments. ND = not determined.

Influence of Retinaldehyde on Formation of Uric Acid by Xanthine Oxidase

The effect of retinaldehyde on XO activity was studied in assays in which the conversion of xanthine to uric acid occurred, at a 1–4 μM fixed all *trans*-retinaldehyde concentration. In these conditions a 15–44% inhibition of XO activity was observed. The K_i for all *trans*-retinaldehyde was determined to be 3 ± 0.4 μM as shown in Figure 7, where competitive inhibition is seen.

Retinoid Content in Bovine Milk

By determination of retinoid concentrations in bovine milk, as reported in Table III, an orientational comparison was made between milk retinaldehyde content (0.45 μM) and the

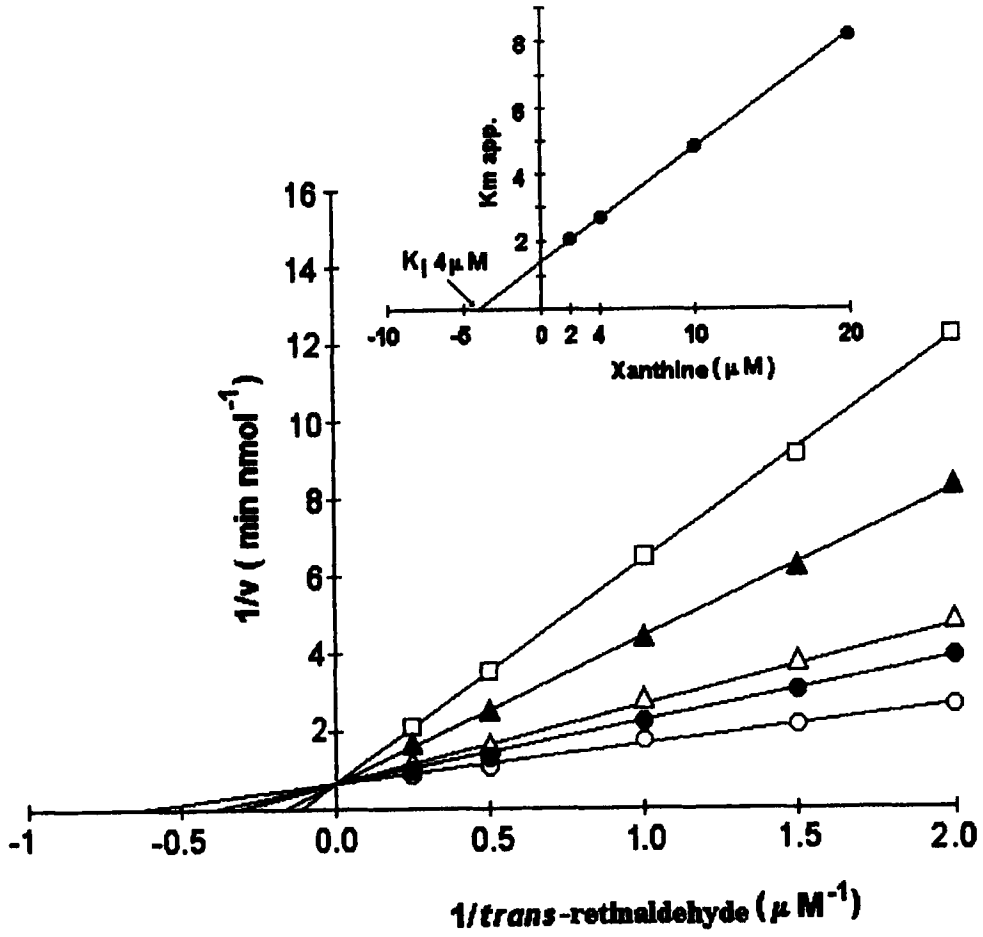

FIGURE 6 Plot of inhibition by xanthine on xanthine oxidase catalysing the oxidation of all *trans*-retinaldehyde to all *trans*-retinoic acid. The base activity was assayed with 1 nM enzyme in the range of retinaldehyde concentration of 0.5–4 μM (○). The xanthine concentrations were fixed at 2 μM (●), 4 μM (△), 10 μM (▲), or 20 μM (□). The plot is representative of four experiments. The K_m replot versus xanthine concentrations shows the inhibition constant value for xanthine.

retinaldehyde K_m value (0.29 μM) determined for XO acting as the retinaldehyde oxidase system, at the lowest O_2 concentrations assayed.

DISCUSSION

Milk xanthine oxidase showed a significant ability to convert retinaldehyde to retinoic acid. This observation stimulated us to seek to define the conditions under which the enzyme can best act *in vitro* to catalyze the biosynthesis of retinoic acid. To this end we considered the effect of changes in concentrations of retinaldehyde and oxygen and the influence of possible cofactors, and so we determined specific activity, turnover number and kinetic parameters for this enzyme. The value of 31.56 min^{-1} determined for the K_{cat} of XO using all *trans*-retinaldehyde is quite high considering the hydrophobic nature of this substrate. The observed value of K_{cat} for XO using the first and best-known substrate of the enzyme is 850–1100 min^{-1} at pH 8.5;[28,29] under our assay conditions, at the same pH value, it was found to be 1864 min^{-1}. For XO (1 nM) oxidizing all *trans*-retinaldehyde at pH 7.0 we determined

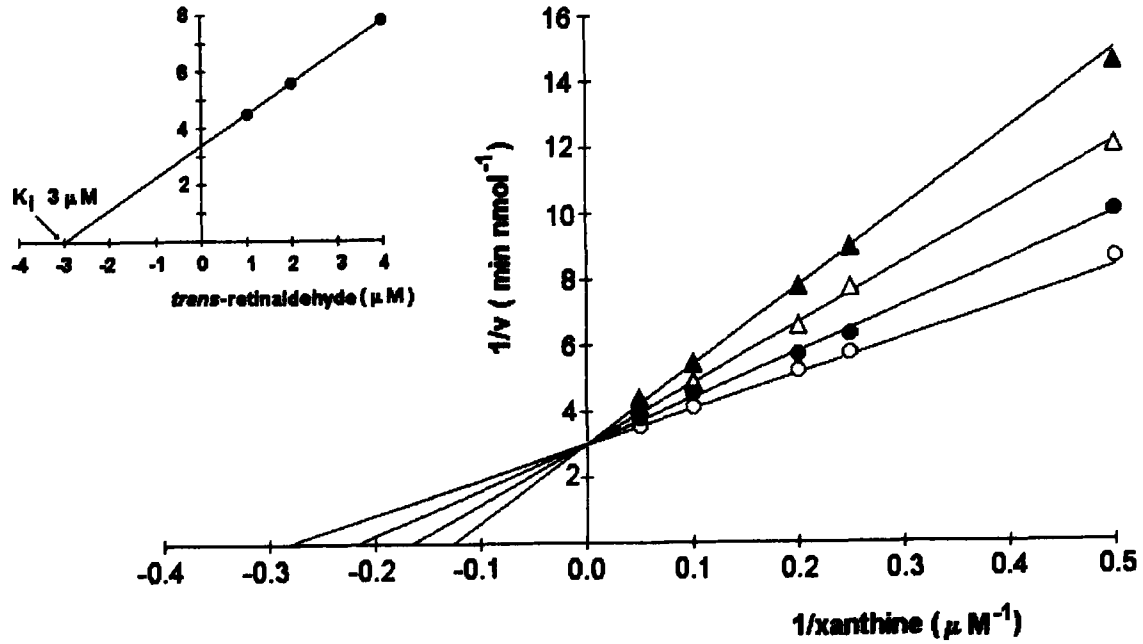

FIGURE 7 Plot of inhibition by all *trans*-retinaldehyde on xanthine oxidase forming uric acid from xanthine. The base activity was assayed with 0.25 nM enzyme in the range of xanthine concentrations of 2–20 μM (○). The retinaldehyde concentration was fixed at 1 μM (●), 2 μM (△), or 4 μM (▲). Data are the mean of three separate experiments. The K_m replot versus retinaldehyde concentrations shows the inhibition constant value for retinaldehyde.

a specific activity of 105 nmol min^{-1} mg^{-1}, while 27 nmol min^{-1} mg^{-1} was the specific activity determined by Futterman[3] assaying 1.55 μM milk XO with 1.25 mM all *trans*-retinaldehyde, a markedly higher value than that of purified liver aldehyde oxidase determined by this author under similar experimental conditions. A value of 267 nmole min^{-1} mg^{-1} was reported by Labrecque *et al.*[20] for the purified kidney retinal dehydrogenase operating at retinaldehyde concentrations in the range 1.5–13 μM. A K_{cat} of 57 min^{-1} can be calculated from activity data reported by Posch *et al.*[19] for the rat liver purified cytosolic dehydrogenase tested with free all *trans*-retinaldehyde. In mouse liver, the dehydrogenase form of XO has been considered to contribute only 4.2% to the catalysis of retinaldehyde oxidation, the rest being accounted for by the isoform ADH_2 of aldehyde dehydrogenase.[16]

TABLE III Retinoid content in bovine milk

	μM[†]
retinaldehyde	0.450 ± 0.062
retinoic acid	0.013 ± 0.004
retinol	1.080 ± 0.095
retinyl esters	0.365 ± 0.075

[†]The values relate to seven determinations (mean ± SD). Extraction of retinoids was performed on fresh bovine milk. Retinoid concentration was determined by HPLC analysis, as reported in Experimental.

The K_m values 290–220 nM we determined for all *trans*-retinaldehyde at different increasing oxygen concentrations are higher than the concentration of this substrate in the cell,[18] but similar[19] or higher values have been reported for several aldehyde and alcohol dehydrogenases involved in retinoic acid biosynthesis.[8,9,13,22,38,39]

The parameters reported here were determined by following the retinaldehyde oxidase activity in assays where retinaldehyde and oxygen were the only substrates added to XO. Indeed, NAD addition, although increasing the V_{max} and K_m values, resulted in non-saturating kinetics in the retinaldehyde concentration range

0.05–2 μM and caused a decrease in the enzyme catalytic efficiency, as measured by the K_{cat}/K_m ratio. It is known that XOs from rat liver and bovine milk react rapidly with oxygen but not with NAD, unlike XDHs from chicken and rat livers and from bovine milk, which react rapidly with NAD but slowly with oxygen. These differences in structure and properties of XDH and XO[40,41] have been reviewed by Saito and Nishino,[42] Nishino,[43] and Hunt and Massey.[44]

The inhibition exerted by xanthine on the retinaldehyde oxidase reaction of XO might be of limited importance *in vivo*, given the fast metabolic fluctuations of purines.[45] In milk serum the following concentrations for the secreted purines were determined: hypoxanthine 54 ± 4.8 μM, xanthine 7.0 ± 0.5 μM and uric acid 153 ± 12 μM. The inhibitory effect of allopurinol on retinaldehyde oxidase activity was also taken into account in order to indirectly deduce that the mechanism of the reaction of retinaldehyde oxidation by XO is probably the same as that for purine substrates.

The inhibitory effect of retinaldehyde on xanthine oxidation and that of xanthine on retinaldehyde oxidation seem to be similar *in vitro*. However, since the retinaldehyde content in the cell is lower than that of xanthine, we deduce that the oxidation of retinaldehyde by XO will be possible at low xanthine concentrations while the inhibitory effect of retinaldehyde on the conversion of xanthine to uric acid is probably not physiologically feasible.

In summary, such reciprocal interferences between retinaldehyde and xanthine interacting with XO might be less significant *in vivo* and might be dependent on the variations in xanthine and retinaldehyde concentrations in the cell. In this study, because of the simultaneous transformation of these substrates by XO, their true concentration in inhibition assays is not the declared one. Hence, the inhibition parameters determined for retinaldehyde or xanthine are apparent. Furthermore, because xanthine is more rapidly transformed than retinaldehyde by XO, the measured $K_{i\,xanthine}$ will be a more approximate value than $K_{i\,retinaldehyde}$.

An interesting aspect of the catalysis of retinaldehyde to retinoic acid by XO is the enzyme's ability to process all retinaldehyde isomers, albeit at different rates. Elucidation of the synthetic pathways to retinoic acids is important as these are ligands for RAR and RXR receptors.[46,47] Isomerization of all *trans*-retinoic acid to 9-*cis*-retinoic acid or 13-*cis*-retinoic acid catalysed by bovine liver microsomes has been described by Urbach and Rando.[48] The same authors consider that another way to generate 9-*cis*-retinoic acid is by the cleavage of 9-*cis*-β-carotene, as already considered,[49] and as also demonstrated for all *trans*-retinoic acid from β-carotene[50] in various tissues via β-apocarotenoic acid.[51] Actually, a variety of all *trans*- and *cis*-carotenoids in human tissues is available.[52] Moreover, a stereospecific 9-*cis*-retinol dehydrogenase has been expressed in CHO cells encoded by a cDNA clone from human breast.[53] An aldehyde dehydrogenase isoenzyme which participates in the catalysis of 9-*cis*-retinaldehyde to the corresponding retinoic acid is active in the rat kidney[38] and alcohol dehydrogenases class I in liver and class IV in epithelia and mucosa oxidize 9-*cis*-retinaldehyde.[39] As mentioned in the introduction, we believe that XO-catalysed retinaldehyde oxidation is of physiological importance and so we evaluated retinoid concentration in bovine milk. Despite the large concentration of XO in the milk-fat-globule membrane,[54,55] secreted by mammary cells, and the presence of retinoids in milk (total content in milk about 1.9 μM), it is by no means obvious that one should correlate these data with the conditions in the cell. We believe, however, that a comparison between the retinaldehyde content (0.45 μM) in milk and the K_m value (0.29 μM) determined for retinaldehyde in this study may indicate that the processing of all *trans*-retinaldehyde *in vitro* by XO is, *in vivo*, a real process.

From these data we would like to suggest that XO should be considered as more than a putat-

ive enzyme for the biosynthesis of retinoic acid and to emphasize its multifarious biological activities.

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