Milk Xanthine Oxidase Type D (Dehydrogenase) and Type O (Oxidase)

PURIFICATION, INTERCONVERSION AND SOME PROPERTIES

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1. The xanthine oxidase of cow's milk, crude or purified, appears as an oxidase (type O), and can be converted almost completely into a NAD^+ -dependent dehydrogenase (type D) by treatment with dithioerythritol or dihydrolipoic acid, but only to a small extent by other thiols. 2. The D form of the enzyme is inhibited by NADH, which competes with NAD^+ . 3. The kinetic constants of the two forms of the enzyme are similar to those of the corresponding forms of rat liver xanthine oxidase. 4. Milk xanthine oxidase is converted into an irreversible O form by pretreatment with chymotrypsin, papain or subtilisin, but only partially with trypsin. 5. The enzyme as purified shows a major faster band and a minor slower band on gel electrophoresis. The slower band is greatly reinforced after xanthine oxidase is converted into the irreversible O form by chymotrypsin.

The xanthine oxidase (xanthine–oxygen oxidoreductase, EC 1.2.3.2) of rat liver supernatant actually appears as a NAD^+ -dependent dehydrogenase (called type D), and is converted into an oxidase (type O) by various treatments (Stirpe & Della Corte, 1969). These treatments may be restricted to two categories, (I) proteolysis, and (II) oxidation or binding of thiol group(s). Only the effect of the treatments of category (II) can be reversed, since the xanthine oxidase type O obtained in this way is reconverted into type D by dithioerythritol (Della Corte & Stirpe, 1972). It was observed also that the xanthine oxidase from mouse duodenum (Joyce & Duke, 1971) and from various rat organs (Battelli *et al.*, 1972) can be obtained also as a dehydrogenase, if the activity of proteolytic enzymes and the oxidation of thiols was prevented during the preparation of the organ extracts.

The present paper describes the purification of xanthine oxidase from cow's milk: the enzyme is obtained in a reversible oxidase form, which can be converted into a dehydrogenase by treatment with dithioerythritol, or into an irreversible oxidase form by proteolysis. Some kinetic and other properties of the enzyme have also been studied.

Experimental

Chemicals

Xanthine (monosodium salt), trypsin (type XI), α -chymotrypsin (type II), papain, subtilisin (type VII), soya-bean trypsin inhibitor, GSH, dithioerythritol, DL- α -lipoic acid (reduced form), D-penicillamine hydrochloride, cysteamine and bovine serum albumin were from Sigma Chemical Co., St. Louis, Mo., U.S.A.; NAD^+ and lactate dehydrogenase

were from Boehringer und Soehne G.m.b.H., Mannheim, Germany; L-cysteine hydrochloride and 2-mercaptoethanol were from E. Merck A.-G., Darmstadt, Germany; DL-homocysteine was from Fluka A.-G., Buchs, Switzerland; thiola (2-mercapto-propionylglycine) was from K & K Laboratories Inc., Plainview, N.Y., U.S.A. Purified milk xanthine oxidase prepared by the method of Gilbert & Bergel (1964) was obtained from Miles-Seravac Ltd., Maidenhead, Berks., U.K. All other chemicals were of analytical grade.

Assay of xanthine oxidase activity

The xanthine oxidase activity was determined spectrophotometrically at 25°C by following the amount of uric acid formed (from the E_{292}), without NAD^+ (oxidase activity) and in the presence of NAD^+ (oxidase plus dehydrogenase activity), as described by Stirpe & Della Corte (1969). The formation of NADH was measured from the E_{340} . Initial rates were measured in all experiments. In some cases pyruvate dehydrogenase and lactate dehydrogenase were added to reoxidize the NADH formed which inhibits xanthine dehydrogenase (Della Corte & Stirpe, 1970, and present experiments). The xanthine oxidase activity is expressed in units corresponding to 1 μmol of product (uric acid or NADH) formed/min.

Protein was determined by the method of Gornall *et al.* (1949) or Lowry *et al.* (1951) with bovine serum albumin as a standard.

K_m values were calculated from Lineweaver–Burk plots, lines being fitted by the least-squares method. K_i values were calculated from the formula given by Dixon & Webb (1964) for competitive inhibitors.

Gel electrophoresis

Disc electrophoresis on polyacrylamide gels was performed by the method of Davis (1964). Purified xanthine oxidase was freed of $(\text{NH}_4)_2\text{SO}_4$ by overnight dialysis against 0.2M-sodium phosphate buffer, pH 6.0, containing 1mM-EDTA, 1.25mM-sodium salicylate and 10mM-2-mercaptoethanol. In early experiments samples preincubated with chymotrypsin were freed from the latter by passing them through a small column of calcium phosphate gel, and were then concentrated by precipitation with $(\text{NH}_4)_2\text{SO}_4$ (60% saturation). The samples were dialysed against 0.05M-Tris containing 0.38M-glycine and adjusted to pH 8.3 with HCl, and were concentrated by dialysis against dry sucrose. The first dialysis and the calcium phosphate step were omitted in subsequent experiments (including that reported in Fig. 5) since control runs showed that chymotrypsin was not visible on the gels. Samples (5 μl) containing approx. 50 μg of protein were used for each electrophoresis. Electrophoresis was done at 2.5mA per tube at room temperature for 2.5–3 h. Gels were stained for protein with Amido Schwarz or with Coomassie Blue.

Results

Purification of xanthine oxidase

The enzyme was purified by a modification of the procedures of Gilbert & Bergel (1964) and of Massey *et al.* (1969). Milk or buttermilk were used as sources of the enzyme. As soon as the cow had been milked, the milk was refrigerated and 2-mercaptoethanol (10mM final concn.), EDTA (1mM final concn.) and sodium salicylate (1.25mM final concn.) were immediately added. The milk was centrifuged for 1 h at 75000g in a Beckman-Spinco L2-65K centrifuge with rotor no. 30. The semi-solid precipitate and the fat floating on the top were discarded, and the liquid part was collected and treated as described below for buttermilk.

Buttermilk was obtained from a dairy, and 2-mercaptoethanol, EDTA and sodium salicylate were added at the same concentrations used for milk. The following operations were performed at 4°C. Butanol (167ml/l of buttermilk), previously cooled at –20°C, and solid $(\text{NH}_4)_2\text{SO}_4$ (194g/l) were added with constant stirring. Stirring was continued for a further 30 min; the mixture was left in the cold for 2.5 h when milk was the starting material, or overnight when buttermilk was used (this difference was due to practical reasons, depending on the time of delivery of milk or buttermilk). After centrifugation for 20 min at 1000g, the aqueous bottom phase was collected, and $(\text{NH}_4)_2\text{SO}_4$ (110g/l) was added with stirring. After 90 min in the cold, the bottom phase was discarded, and the top phase was centrifuged for 30 min at 10000g. The top phase formed after

Table 1. *Purification of xanthine oxidase*

The enzyme activity was measured as described by Stirpe & Della Corte (1969). The reaction mixture contained, in a final volume of 3 ml: 0.1 M-Tris-HCl buffer, pH 8.1, 60 μ M-xanthine, 0.67 mM-NAD⁺ (when present) and 0.1 ml of suitably diluted enzyme. Xanthine was omitted from reference cuvettes. Formation of uric acid at 25°C was measured at 292 nm, and formation of NADH at 340 nm.

Step	Assayed with	Volume (ml)	Concn. of enzyme (munits/ml)		Total enzyme activity (units)				Specific enzyme activity (munits/mg of protein)				Yield (%) [*]	Relative purification [*]
			O ₂	O ₂ +NAD ⁺	O ₂	Uric acid	NADH	Uric acid	NADH	Protein (mg/ml)	Uric acid	NADH		
Buttermilk	...	307	119.6	123.4	19.4	36.7	37.9	0.1	11.7	10.2	10.5	1.7	100	1
35%-satd. (NH ₄) ₂ SO ₄	...	285	122.8	127.2	35.3	35.0	36.2	10.1	1.9	64.6	66.9	18.6	96	6.4
Diffusate	15.6	2031	1813	106.5	31.7	28.3	1.7	13.3	13.3	152.7	136.3	8.0	75	13
Column eluate	17	1234	1081	58.1	21.0	18.4	1.0	1.0	1.0	1234	1081	58.1	48	103
Final preparation	1.5	12230	12886	1446	18.3	19.3	2.2	7.7	7.7	1588	1673	187.8	51	159

^{*} Calculated from the activity with O₂ + NAD⁺.

centrifugation was collected and resuspended in a small volume (20 ml/l of buttermilk) of 0.2M-sodium phosphate buffer, pH 6.0, containing mercaptoethanol, EDTA and sodium salicylate at the concentrations described above. The suspension was dialysed overnight against the same buffer, and the precipitate remaining after dialysis was removed by centrifugation at 60000g for 20 min. The clear supernatant was passed through a column of calcium phosphate gel mixed with cellulose (10 g of gel per litre of buttermilk, plus 15 g of cellulose), previously equilibrated with the phosphate buffer containing 2-mercaptoethanol, EDTA and sodium salicylate. The column was washed with the same buffer, and the E_{253} of the effluent was monitored. After the bulk of protein was washed out, the column was eluted with the same buffer containing 5% (w/v) $(\text{NH}_4)_2\text{SO}_4$, at the rate of 30 ml/h. Fractions of 5–6 ml were collected. The xanthine oxidase was eluted as a sharp peak in two to three fractions. These were pooled, and the enzyme was precipitated by making the solution 60% saturated with $(\text{NH}_4)_2\text{SO}_4$. The precipitate was collected by centrifugation, and resuspended in a small volume (2 ml/l of buttermilk) of the sodium phosphate buffer, 60% saturated with $(\text{NH}_4)_2\text{SO}_4$. The enzyme was stored at 2°C, and was stable for months. A summary of the purification procedure is given in Table 1. The recovery and the characteristics of the enzyme were the same if milk or buttermilk was used as starting material. Although a higher content of enzyme was found in the 'buttermilk' obtained by centrifugation of milk, the dairy buttermilk was used for preparing xanthine oxidase as a routine, since it was easier to handle, especially when large amounts were used.

Conversion of xanthine oxidase into a dehydrogenase

The xanthine oxidase purified as described above appeared as an oxidase, since its activity was not stimulated by the addition of NAD^+ . However, if NAD^+ was present in the assay mixture, some NADH was formed, thus indicating that at least part of the enzyme was in the postulated 'intermediate' form, which may react with O_2 as well as with NAD^+ (Della Corte & Stirpe, 1972). This formation of NADH was decreased or abolished after the enzyme had been preincubated at 37°C for 10–20 min, whereas the activity measured from the formation of uric acid was not affected, or was slightly increased.

The xanthine oxidase was converted into a dehydrogenase by preincubation at 37°C in the presence of 10 mM-dithioerythritol. The conversion occurred in 10–20 min (Fig. 1) and was accompanied by some loss of enzyme activity, similar to that observed with the rat liver enzyme (Della Corte & Stirpe, 1972). The enzyme retained some oxidase activity, which did not

Fig. 1. Conversion of xanthine oxidase into a dehydrogenase by preincubation with dithioerythritol

The enzyme was preincubated at 37°C in 0.2M-tris-HCl buffer, pH 8.1, with 10 mM-dithioerythritol. Assay conditions were as described in Table 1. ●, Uric acid formed with O_2 as acceptor; ▲, uric acid formed, and ■, NADH formed, with $\text{O}_2 + \text{NAD}^+$ as acceptors. —, Enzyme preincubated with dithioerythritol; ----, enzyme preincubated without dithioerythritol.

disappear (and actually increased) if the preincubation at 37°C was prolonged.

Various thiols were tested as possible converting agents (Table 2): only dihydrolipoic acid had an effect comparable with that of dithioerythritol, although a dose-response curve showed that the latter thiol was effective at lower concentrations than dihydrolipoic acid (Fig. 2).

Commercially obtained milk xanthine oxidase was not converted into a dehydrogenase by dithioerythritol.

Conversion of xanthine oxidase into an irreversible oxidase form by proteolytic enzymes

The effect of various proteolytic enzymes on milk xanthine oxidase was tested in the presence of dithioerythritol. The enzyme was completely converted into the O form by chymotrypsin, papain and subtilisin (Table 3), whereas trypsin brought about only

Table 2. Conversion of xanthine oxidase from the O into the D form by various thiols

Enzyme assays were performed as described in Table 1, the purified enzyme being used. The enzyme was preincubated at 37°C for 10min with thiols (20mM final concn., except for dithioerythritol and dihydrolipoic acid, which were 10mM) in 0.2M-Tris-HCl buffer, pH8.1.

Expt no.		Additions	Xanthine oxidase activity (units/ml)	Xanthine oxidase activity (units/ml)		
				O ₂	NAD ⁺ +O ₂	
					Uric acid formed	NADH formed
1	Not preincubated	None	12.2		12.2	1.1
	Preincubated for 10min at 37°C	None	12.1		11.7	0.5
		Dithioerythritol	4.7		10.8	7.3
		Cysteine	9.4		11.7	3.6
		Homocysteine	8.2		11.8	4.8
		Cysteamine	11.5		11.1	2.2
		GSH	8.9		11.5	4.4
		Mercaptoethanol	9.4		11.2	4.4
		Dihydrolipoic acid	3.3		9.6	8.7
		Penicillamine	12.9		13.1	0.8
		Thiola	9.8		11.9	3.7
2	Not preincubated	None	13.3		13.4	1.1
	Preincubated for 10min at 37°C	None	12.8		13.0	0.8
		Dithioerythritol	5.0		11.6	8.1
		Dihydrolipoic acid	3.8		10.9	9.3

Fig. 2. Conversion of xanthine oxidase into a dehydrogenase by preincubation with various concentrations of dithioerythritol or dihydrolipoic acid

Experimental conditions were as in Fig. 1 except for the concentration of dithioerythritol. Preincubation was for 10min with dithioerythritol (a) or with dihydrolipoic acid (b). ●, Uric acid formed with O₂ as acceptor; ▲, uric acid, and ■, NADH, formed with O₂+NAD⁺ as acceptors.

Table 3. *Conversion of xanthine oxidase into the irreversible O form by proteolytic enzymes*

Experimental conditions were as described in Table 2. Xanthine oxidase was preincubated at 37°C for 45 min with the proteolytic enzymes (0.5 mg/ml) in 0.1 M-Tris-HCl buffer, pH 8.1, in the presence of 10 mM-dithioerythritol.

Additions	Acceptor ...	Xanthine oxidase activity (units/ml)		
		O ₂	O ₂ +NAD ⁺	
			Uric acid formed	NADH formed
None		1.35	3.05	1.90
Trypsin		2.65	3.40	0.90
Chymotrypsin		3.30	3.30	0.05
Papain		3.40	3.40	0.05
Subtilisin		3.10	3.00	0.10

a partial conversion, as indicated by the stimulation of uric acid formation in the presence of NAD⁺, and by the formation of NADH. A complete conversion could not be obtained with trypsin by prolonging the incubation, by omitting dithioerythritol or by adding xanthine, which facilitated the effect of trypsin on the rat liver xanthine oxidase (Stirpe & Della Corte, 1969).

The effect of various concentrations of chymotrypsin and of the presence of dithioerythritol is shown in Fig. 3. The conversion was complete in the presence of lower amounts of chymotrypsin if dithioerythritol was omitted, whereas higher concentrations were required if the thiol was added at the same time as chymotrypsin. The conversion was not complete (within the range of concentrations tested) if xanthine oxidase was treated with dithioerythritol before proteolysis, i.e. if the enzyme was previously converted into the dehydrogenase form.

Properties of the purified xanthine oxidase

Kinetic properties. The kinetic constants of the various forms of xanthine oxidase are reported in Table 4. The K_m (xanthine) value of the O form and the K_m (NAD⁺) value of the D form were very close to the corresponding constants of the rat liver enzyme, whereas the K_m (xanthine) value of the D form was slightly higher (Della Corte & Stirpe, 1972). The D form was inhibited by NADH, which acted by competing with NAD⁺ (Fig. 4), with a K_i value very close to that of the rat or chick liver xanthine dehydrogenase (Della Corte & Stirpe, 1970).

Gel electrophoresis. Xanthine oxidase subjected to gel electrophoresis as prepared showed a main band migrating toward the cathode, and a second slower band, which sometimes was scarcely visible (Fig. 5). The same pattern was observed if the enzyme was

Fig. 3. *Conversion of xanthine oxidase into the irreversible oxidase form by chymotrypsin*

Preincubations were at 37°C in 0.1 M-Tris-HCl buffer, pH 8.1. ●, ■, ▲, Uric acid formed with O₂ as acceptor; ○, □, △, NADH formed with O₂ + NAD⁺ as acceptors. ●, ○, The enzyme was preincubated with chymotrypsin for 45 min, then dithioerythritol (10 mM final concn.) was added, and preincubation was continued for a further 20 min. ■, □, The enzyme was preincubated for 45 min with chymotrypsin in the presence of 10 mM-dithioerythritol. ▲, △, The enzyme was preincubated for 20 min in the presence of 10 mM-dithioerythritol, then chymotrypsin was added and preincubation was continued for further 45 min.

Table 4. *Kinetic constants of the O and D types of milk xanthine oxidase*

Xanthine oxidase type O (reversible) was the enzyme as prepared. The enzyme type O (irreversible) was obtained by preincubation at 37°C for 45 min with chymotrypsin (0.5 mg/ml) in 0.1 M-Tris-HCl buffer, pH 8.1, in the presence of 10 mM-dithioerythritol. The enzyme type D was obtained by preincubation at 37°C for 20 min with 10 mM-dithioerythritol in 0.2 M-Tris-HCl buffer, pH 8.1. Values obtained without NAD⁺ (residual oxidase activity) were subtracted from the values obtained with the enzyme type D assayed in the presence of NAD⁺. Sodium pyruvate (5 μ mol) and lactate dehydrogenase (10 μ l of a 1:10 dilution of the enzyme) were added to the samples without NADH.

Enzyme	Acceptor	Final concn. of NADH added (nM)	$10^6 \times K_m$ (xanthine) (M)	$10^6 \times K_m$ (NAD ⁺) (M)	$10^6 \times K_t$ (NADH) (M)
Type O (reversible)	O ₂	—	5.65	—	—
Type O (irreversible)	O ₂	—	4.24	—	—
Type D	NAD ⁺	—	12.1	15.3	—
	NAD ⁺	5	—	33.8	2.58
	NAD ⁺	20	—	133.4	2.59

Fig. 4. *Inhibition of xanthine oxidase type D by NADH*

The enzyme was converted into the D form by preincubation at 37°C for 20 min with 10 mM-dithioerythritol in 0.2 M-Tris-HCl buffer, pH 8.1. The enzyme was assayed as described in Table 1 without NADH (●), with 5 μ M-NADH (○) and with 20 μ M-NADH (Δ). Sodium pyruvate and lactate dehydrogenase were added to samples without NADH (see Table 4). v is expressed in arbitrary units ($10^3 \times \Delta E_{292}/\text{min}$ per sample containing 19 μ g of protein).

treated with dithioerythritol before electrophoresis. However, it should be pointed out that the enzyme treated in this way appeared as an oxidase when assayed immediately before electrophoresis. Presumably the dehydrogenase form had been reconverted into the original reversible oxidase form during dialysis and/or other manipulations, and consequently the results obtained with these preparations are not shown.

Fig. 5. *Gel electrophoresis of xanthine oxidase*

The electrophoresis was performed as described in the Experimental section with xanthine oxidase in the reversible O form (native enzyme, a) and in the irreversible O form (b). The latter form was obtained by preincubation of the enzyme at 37°C for 45 min with chymotrypsin (10 μ g/ml). Migration was from anode (top) to cathode (bottom). Starting point is indicated by the arrow.

The electrophoresis of xanthine oxidase treated with chymotrypsin showed in some cases two bands corresponding to those of the native preparations, but approximately of the same intensity, owing to a considerable reinforcement of the slower-migrating band. In other instances, as in the experiment reported in Fig. 5, the faster band disappeared, and only the slower band could be seen after treatment with chymotrypsin.

Discussion

The procedure described in this paper makes it possible to purify milk xanthine oxidase in a form that can be almost completely converted into a NAD^+ -dependent dehydrogenase. This method may be adapted to larger volumes, thus allowing the preparation of larger quantities of enzyme from a source material more easily available than rat liver.

Unlike the enzyme from rat organs (Stirpe & Della Corte, 1969; Joyce & Duke, 1971; Battelli *et al.*, 1972), milk xanthine oxidase could not be obtained as a dehydrogenase, but appeared as a reversible oxidase even when buttermilk was obtained in the laboratory from milk to which mercaptoethanol was added immediately after milking. It is possible that the enzyme is produced in this reversible O form, but the possibility should be considered that the D form is converted into the reversible O form while milk is 'stored' in mammary glands. In any case, it can be concluded that the interconvertibility between D and O forms is a common property of the xanthine oxidase from all mammalian sources investigated so far [rat organs (Stirpe & Della Corte, 1969; Joyce & Duke, 1971; Battelli *et al.*, 1972); human liver (Della Corte *et al.*, 1969); cow's milk (present experiments)]. Apart from its original form, milk xanthine oxidase behaves largely as the enzyme from rat liver, and the kinetic properties of the two enzymes are similar (Della Corte & Stirpe, 1972). It is noteworthy that the O form can be converted into the D form on treatment with dihydrolipoic acid, which may play a role in the possible interconversion of the two forms of the enzyme under physiological conditions.

Milk xanthine oxidase is converted into the irreversible O form by several proteolytic enzymes, though trypsin has only a limited effect. This is another difference between milk and rat liver xanthine oxidase, and may be useful to elucidate the modifications underlying the conversion of the enzyme from the D into the O form. It is also noteworthy that the conversion into the irreversible O form brought about by chymotrypsin was more difficult in the presence of dithioerythritol, and even more if the enzyme was pretreated with the thiol, i.e. was in the D form. The fact that the reversible O form is converted more easily than the D form suggests that the

reversible O form may be an intermediate stage in the conversion of xanthine oxidase between the D and irreversible O form, as proposed by Della Corte & Stirpe (1972). Commercially obtained xanthine oxidase cannot be converted into a dehydrogenase since the purification procedure included treatment with a proteolytic enzyme.

Our present results and those obtained with the rat liver enzyme (Della Corte & Stirpe, 1972) can be compared with the results of Komai *et al.* (1969). These investigators observed that removal of flavin from milk xanthine oxidase brought about the loss of the xanthine-oxygen oxidoreductase activity, although the enzyme was still reduced by xanthine, and its xanthine-ferricyanide reductase and xanthine-cytochrome *c* reductase activities were increased. The enzyme they used had been prepared by a procedure involving pancreatin digestion (Massey *et al.*, 1969) and therefore was presumably in the irreversible O form, hence unable to reduce NAD^+ . If this is so, then on the basis of the model proposed by Komai *et al.* (1969) and of our results, a working hypothesis may be formulated: (i) that NAD^+ is reduced at a different binding site on xanthine oxidase from ferricyanide and cytochrome *c*, and (ii) that one or more thiol group(s) are required for the enzyme to react with NAD^+ , and at the same time they prevent the reaction with O_2 , possibly by masking or influencing in other ways the reactivity of FAD, which is present in our enzyme preparations. The mechanism whereby proteolysis brings about similar (although irreversible) changes to those caused by thiol oxidation remains to be explained, but it is possible that proteolysis breaks down a fragment containing or affecting in some way the essential thiol group(s).

The preparations of xanthine oxidase in the 'native' conditions showed two bands on gel electrophoresis. The slower band (usually smaller) increased, and the faster one decreased or disappeared after conversion of the enzyme into the irreversible O form by chymotrypsin. It seems that the faster and the slower band are, respectively, the reversible and irreversible O forms of the enzyme. This is not consistent with the results of Joyce & Duke (1971), who observed that mouse liver xanthine oxidase treated with trypsin had a higher electrophoretic mobility than the untreated enzyme. On the other hand, it is possible that the enzyme is converted into the irreversible O form after part of its molecule is removed by chymotrypsin. In either case, the presence of the slower band in the untreated preparations of xanthine oxidase suggests that these preparations contain some enzyme in the irreversible O form, and this accounts for the residual oxidase activity after treatment with dithioerythritol.

Procedures avoiding the use of proteolysis have been used by other investigators to prepare xanthine oxidase (Nelson & Handler, 1968; Massey *et al.*, 1969; Hart *et al.*, 1970). When the results obtained by

these methods are compared with ours, it appears that our procedure gives an enzyme with lower specific activity, which at least in some cases can be accounted for by the considerable experimental differences (Nelson & Handler, 1968; Hart *et al.*, 1970). On the same basis one could explain the homogeneity of the enzyme prepared by Hart *et al.* (1970), whereas the enzyme prepared by Nelson & Handler (1968) showed some impurities when examined in the ultracentrifuge. The procedure followed in the present work is very similar to that of Massey *et al.* (1969), who also obtained an enzyme of higher specific activity. Our results are somewhat variable and sometimes we obtain preparations with a specific activity 3–4 times higher than that reported in Table 1. The enzyme prepared without proteolysis by Massey *et al.* (1969) was homogeneous on gel electrophoresis. If the slower band present in our preparations is the irreversible O form of xanthine oxidase, the difference may be due to conversion of some enzyme occurring to a variable extent during the preparation, possibly owing to some uncontrolled factor(s).

Note added in proof (received 27 November 1972)

After this had been accepted, we observed that buttermilk kept at 4°C for 4 days had a higher apparent xanthine oxidase activity and gave a higher yield of purified enzyme.

The following results were obtained in two separate experiments (data are in units, and are referred to 1 litre of buttermilk): total activity of buttermilk: fresh, 36.9 and 40.9; stored, 46.4 in both experiments; total activity recovered: from fresh buttermilk, 9.46 and 12.6; from stored buttermilk, 14.2 and 21.1.

The purified enzyme appeared in each case as an oxidase, and could be converted into a dehydrogenase as described in the paper.

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