Xanthine Oxidase

Hans Lück

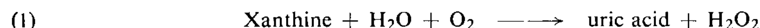
Xanthine oxidase*) is a flavoprotein containing iron and molybdenum, which occurs in milk, in some organs and tissues and in bacteria. It catalyses the oxidation of hypoxanthine and xanthine (hydrated) to uric acid. Its specificity is low: it also oxidizes other purines as well as aliphatic and aromatic aldehydes. The hydrogen acceptors are O_2 ($\rightarrow H_2O_2$), redox dyes or cytochrome c. For the chemistry and biochemistry of xanthine oxidase, see [1–3].

Xanthine oxidase activity can be directly measured by the determination of the reaction products, *e.g.* uric acid[4]. More usual methods are the manometric determination of the oxygen consumed[5,6] or measurement of the colour change occurring on reduction of methylene blue. Other methods are: anaerobic measurement of the change in the redox potential of the system xanthine/xanthine oxidase/methylene blue[7–10]; determination of the amount of formazan formed from triphenyltetrazolium chloride[11–14] or measurement of the reduction of nitrate to nitrite[15,16]; measurement by polarographic analysis[17]; spectrophotometric determination of the amount of reduced cytochrome c formed (only suitable with purified enzyme solutions)[18].

The determination of activity by measurement of the reduction of methylene blue in Thunberg tubes (refer to p. 31) has proved suitable even for turbid samples (milk)[19].

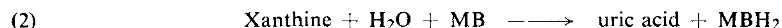
Principle

Xanthine oxidase (XOD) catalyses the reaction:



(1) $$\text{Xanthine} + H_2O + O_2 \longrightarrow \text{uric acid} + H_2O_2$$

Under anaerobic conditions methylene blue (MB) can act as hydrogen acceptor:



(2) $$\text{Xanthine} + H_2O + \text{MB} \longrightarrow \text{uric acid} + \text{MBH}_2$$

The rate of reaction (2) is a measure of the XOD activity. The decrease in the intensity of the colour with time is measured. The MB concentration must be about half that of the xanthine concentration, so that the initial rate of the reaction is easily obtained.

---

*) Synonyms: Xanthine dehydrase, Schardinger enzyme, xanthine-aldehyde-O_2 transhydrogenase. Also in the older literature, aldehyde dehydrase, aldehyde oxidase, aldehyde reductase, aldehyde catalase.

1) *E. C. de Renzo*, Advances in Enzymol. *17*, 293 [1956].
2) *G. Schmidt*, The Nucleic Acids *1*, 609 [1955].
3) *W. Shive:* Vitamins and Hormones *9*, 117 [1951].
4) *H. Wieland* and *B. Rosenfeld*, Liebigs Ann. Chem. *477*, 32 [1929].
5) *E. G. Ball*, J. biol. Chemistry *128*, 51 [1939].
6) *F. L. Rodkey* and *E. G. Ball*, J. Lab. clin. Med. *31*, 354 [1946].
7) *K. Kodama*, Biochem. J. *20*, 1095 [1926].
8) *S. Filitti*, C. R. Séances hebd. Acad. Sci. *197*, 1212 [1933].
9) *D. E. Green*, Biochem. J. *28*, 1550 [1934].
10) *V. H. Booth*, Biochem. J. *29*, 1732 [1935].
11) *E. Shelton* and *W. Schneider*, Anatom. Rec. *112*, 61 [1952].
12) *G. H. Bourne*, Nature [London] *172*, 193 [1953].
13) *H. Wallhäuser*, Naturwissenschaften *38*, 237 [1951].
14) *R. Kuhn* and *D. Jerchel*, Ber. dtsch. chem. Ges. *74*, 949 [1941].
15) *A. Bach*, Biochem. Z. *33*, 282 [1911].
16) *L. M. Buruiana*, Lait *31*, 134 [1951].
17) *E. Knobloch*, Chem. Listy Vědu Průmysl *37*, 10 [1943].
18) *B. L. Horecker* and *L. Heppel*, J. biol. Chemistry *178*, 683 [1949].
19) *F. Kiermeier* and *K. Vogt*, Z. Lebensmittel-Unters. u. -Forsch. *103*, 198 [1956]; *K. Vogt*, Ph. D.-Thesis, Techn. Hochschule München, 1955.

Optimum Conditions for Measurements

The optimum pH is between 6 and 9 with methylene blue as hydrogen acceptor[20]. Outside this range the XOD activity falls off rapidly. Usually pH 7 to 8.5 is used. Excess of the substrate and of the products (uric acid, H_2O_2) inhibit the enzyme. The optimum xanthine concentration is about 10^{-6} moles/assay mixture[19].

Reagents

1. Methylene blue
   dried *in vacuo*; quality "redox indicator"
2. Xanthine
   dried *in vacuo*; commercial preparation, see p. 1033.
3. Potassium dihydrogen phosphate, A. R., KH_2PO_4
4. Disodium hydrogen phosphate, A. R., $Na_2HPO_4 \cdot 2H_2O$
5. Sodium hydroxide, A. R., 0.01 N

Preparation of Solutions

I. Methylene blue (*ca.* 2.5×10^{-4} M):
Stock solution: Dissolve 0.32 g. methylene blue in distilled water and make up to 1000 ml. Methylene blue dissolves very slowly, so allow the stock solution to stand for 1 or 2 days before diluting further (shake occasionally). Just before use dilute the stock solution 1 : 4 with distilled water.

II. Xanthine (5×10^{-4} M):
Stock solution: Dissolve 0.3802 g. xanthine in 0.01 N NaOH and make up to 500 ml. Just before use dilute 1 : 10 with distilled water.

III. Phosphate buffer (0.2 M; pH 7.4):
Dissolve 4.957 g. KH_2PO_4 + 29.144 g. $Na_2HPO_4 \cdot 2H_2O$ in distilled water and make up to 1000 ml.

Stability of the solutions

Solutions II and III are stable for a long period providing no bacterial contamination occurs. The methylene blue solution should be protected from light by storing in a brown bottle and should be freshly prepared each week.

Procedure

Colour standards

Pipette into four test tubes and mix:

	1	*2*	*3*	*4*
sample (heated for a short time at 100°C)	2 ml.	2 ml.	2 ml.	2 ml.
phosphate buffer (solution III)	1 ml.	1 ml.	1 ml.	1 ml.
xanthine solution (II)	1 ml.	1 ml.	1 ml.	1 ml.
methylene blue solution (I)	0.3 ml.	0.2 ml.	0.1 ml.	—
distilled water	0.7 ml.	0.8 ml.	0.9 ml.	1.0 ml.

Tube 4 corresponds to 100% decolorization (in the enzymatic assay), tubes 3, 2 and 1 correspond to 90%, 80% and 70% decolorization respectively.

---

[20] *M. Dixon* and *S. Thurlow*, Biochem. J. *18*, 976 [1924].

Enzymatic assay

Pipette into the main compartment of a Thunberg tube (see p. 31)

2 ml. sample (*e.g.* milk)
1 ml. phosphate buffer (solution III).

Pipette into the stopper:

1 ml. xanthine solution (II)
1 ml. methylene blue solution (I).

Grease the ground-glass joint of the stopper (*e.g.* with "rubber grease") and place in tube. Equilibrate the tube in a water bath at 37°C for *ca.* 20 min. (at 20°C if the enzymic activity of the sample is high). Evacuate *) for about 3 min. on a water pump while shaking gently and then seal the tube by turning the hollow stopper. Tip the contents of the side-arm into the main compartment, start a stopwatch and continue incubation at 37°C. Note the times required for 70%, 80%, 90% and 100% decolorization (visual comparison of the colour with the colour standards).

The time required for complete decolorization should be 5−10 min. If it is shorter dilute the sample correspondingly, and if it is longer prepare greater dilutions of the methylene blue and xanthine stock solutions.

Calculations

According to [21] the activity number k of xanthine oxidase is:

$$k = \frac{10000}{t} \qquad (3)$$

where t is the time (in sec.) required for the complete decolorization of a 10^{-3} M methylene blue solution at 20°C under the conditions described above. Since t increases proportionally with the methylene blue concentration it follows that with a 2.5×10^{-4} M methylene blue solution:

$$k = \frac{10000}{t} \times \frac{2.5 \times 10^{-4}}{10^{-3}} = \frac{2500}{t} \qquad (4)$$

The value for t is obtained graphically: plot the percentage decolorization of the assay mixture against time and draw a line through the points (does not pass through zero). Read off the mean time (in sec.) required for 100% decolorization and insert this value in equation (4).

Example

The following times were measured with 2 ml. raw milk at 20°C:

70%	80%	90%	100%	Decolorization
3.1	3.8	4.3	5.7	min.

The mean time obtained graphically for 100% decolorization was 5.1 min. = 306 sec.

$$k = \frac{2500}{306} = 8.2$$

*) The reproducibility of the results depends considerably on the care taken in evacuation.

[21] *M. Polonovski, E. Neuzil* and *L. Baudu*, Lait *1*, 128 [1947].