

DETERMINATION OF XANTHINE OXIDASE IN MILK WITH  
TRIPHENYL TETRAZOLIUM CHLORIDE

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Xanthine oxidase in crude biological preparations is frequently determined by the methylene blue decolorizing technique. Other more elegant methods are available for purified preparations of soluble xanthine oxidase (5). The methylene blue technique requires the exclusion of oxygen to prevent the spontaneous reoxidation of the reduced, colorless dye. Hence, Thunberg tubes or some similar arrangement must be used to remove the oxygen. Triphenyl tetrazolium chloride (TTC) appeared to be more advantageous for determining xanthine oxidase since its red, reduced form is stable to oxygen. This substance has been used to determine xanthine oxidase in liver tissue (6) and in insects (1). In both these studies the reaction was performed in evacuated tubes. This compound has been used also for the determination of succinic dehydrogenase, and oxygen was found not to interfere seriously (4). A study of the use of TTC for xanthine oxidase in milk has shown that although the reduced compound is stable in air, the enzyme reaction is inhibited by oxygen presumably by competition with the TTC. A simplified technique has been developed, however, in which the enzymatic reaction is carried out in a system freed of air with bubbling nitrogen gas, and subsequent operations are performed in the air. The influence of TTC and substrate concentrations and other factors have also been investigated.

REAGENTS

Triphenyl tetrazolium chloride (TTC), 0.05 *M* 334.5 mg. is dissolved in 20.0 ml. of 0.1 *M* sodium phosphate buffer, pH 7.5.

Xanthine, 0.005 *M*. A 0.05 *M* solution is prepared by dissolving 380.0 mg. in 50.0 ml. of 0.05 *N* NaOH. This stock solution is diluted daily to the required 0.005 *M* concentration.

Phosphate buffer, 0.5 *M*, pH 7.5. Prepared by dissolving 29.1 g.  $\text{Na}_2\text{HPO}_4 \cdot 7 \text{H}_2\text{O}$  and 2.28 g.  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  in water and making the final volume 250 ml. Acetic acid, glacial, C. P.

PROCEDURE

The enzyme xanthine oxidase activates certain hydrogen atoms on xanthine and on a variety of purine and aldehyde substrates with a concurrent reduction of oxygen or of a number of redox compounds. In the present method of determining xanthine oxidase, TTC is reduced by enzyme action to a red product

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which is extracted from the reaction mixture with toluene after acidification with acetic acid. The color in the toluene solution is read in a Beckman type B spectrophotometer<sup>2</sup> in 150 × 18 mm. test tubes at 485 m $\mu$ . The color equivalent of the reduced TTC was obtained by reducing dilute solutions of TTC with sodium hydrosulfite and extracting the color with toluene exactly as in the regular assay procedure. The results were not as reproducible as desired, but an approximate absorbance of 0.36 was obtained with  $3 \times 10^{-7}$  moles (0.6 ml. of 1:100 dilution of 0.05 M TTC) of reduced TTC. If this absorption is corrected for dilution in the toluene, the absorbance is 1.08 for  $3 \times 10^{-7}$  moles (100  $\gamma$ ) of TTC in 4.0 ml. of toluene. This value cannot be compared with other published values (1, 4, 6) because of incomplete description of the optical measurements.

One unit of xanthine oxidase activity is defined as follows: an absorbance of 0.036, equivalent to approximately  $0.3 \times 10^{-7}$  moles of reduced TTC, produced by the enzyme under the specified test conditions.

*Determination of xanthine oxidase in milk.* The enzyme reaction is performed in a water bath at 30° C. in 125 × 15 mm. test tubes. Two determinations are run in parallel with the following reagents present at the start: 1.0 ml. of 0.5 M sodium phosphate buffer, pH 7.5; 0.2 ml. xanthine solution; and water (1.5 ml. in one tube and 1.0 ml. in the other). The bubbling of commercial nitrogen gas into this mixture is begun, through small capillary tubes extending to the bottom of the test tubes, at a rate such that it is impossible to count the bubbles. The capillary tubes are grooved at the end to permit easy escape of the gas. By the use of a manifold as many as six test tubes can be in use simultaneously. The bubbling is continued for 5 minutes and then 0.5 ml. and 1.0 ml. of diluted milk (skim or whole milk 1:5) or cream (1:20) is added to the two tubes. If frothing occurs, it is controlled with a drop of antifoam (5 to 10 mg. Foamrex in 10 ml. water). Bubbling is continued for another 5 minutes. During this period the xanthine oxidase assists enzymatically in removing the last traces of oxygen. Finally, 0.2 ml. of TTC solution is added and the reaction is continued for an accurately timed 10.0 minutes. The final volume is 3.4 ml. The reaction is stopped with the addition of 5.0 ml. of concentrated acetic acid to each tube, and bubbling of the nitrogen is stopped. The capillaries are left in their respective test tubes and used to agitate the 4.0 ml. of toluene, which is added next. Finally, the tubes are stoppered, shaken vigorously, and centrifuged. The red reduced TTC is extracted into the toluene. Two ml. of the colored toluene solution are removed and diluted with 4.0 ml. of toluene. Six ml. of solution are required for the spectrophotometer tube. The colors are read in 150 × 18 mm. test tubes at 485 m $\mu$ .

#### DISCUSSION

The procedure described for the determination of xanthine oxidase in milk was developed after study of a number of factors that are critical for enzyme reactions including concentration of both the substrates and the reagent.

<sup>2</sup> Mention of products does not imply endorsement of the U. S. Department of Agriculture or recommendation over any other products of a similar nature not mentioned.

The optimal concentration of xanthine in the reaction mixture is 0.000088 to 0.00037 mole per liter (0.06 to 0.25 ml. of a 0.005 *M* solution), as shown in Figure 1. At higher concentrations inhibition occurs; for example, a concentration (0.000588 *M*) twice that used in the assay inhibits the reaction by 15%. Inhibition by excess substrate is characteristic of this enzyme (2, 5). Smaller concentrations of xanthine are inadequate for maximum activity. The substrate requirement was studied with an amount of enzyme that gave a maximum

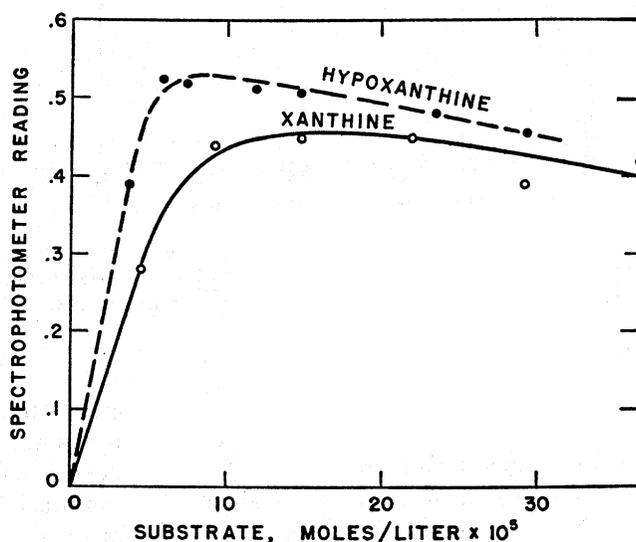


FIG. 1. Effect of xanthine and hypoxanthine concentration on xanthine oxidase activity.

absorbance reading of 0.45 with xanthine. Readings should not exceed 0.6 for the substrate concentration routinely used (0.2 ml. of 0.005 *M*), or the concentration will begin to limit the activity, and the readings and amount of enzyme will not be proportional.

The concentration of TTC required for maximum reactivity is surprisingly high, over a tenfold range of xanthine concentrations, as shown in Figure 2. Methylene blue behaves similarly, but only if the concentration of substrate is high (2). The same concentration dependence of TTC is found with hypoxanthine as the substrate. The concentration of 0.2 ml. of TTC used in the regular assay (final concentration 0.0029 *M*), although below the optimum, is justified since the concentration of TTC changes only about 5% during the reaction.

The pH must be maintained at 7.5 for maximum activity. If acidic undiluted milk samples are assayed, the effectiveness of pH maintenance must be determined. The stability of the xanthine oxidase in milk under the test conditions, particularly after the xanthine was introduced and before the TTC was added, was established by prolonging the bubbling time from 5 to 10 minutes. The results with both bubbling times were the same, indicating that the enzyme was stable during this period. The stability of the enzyme is evident since the amount

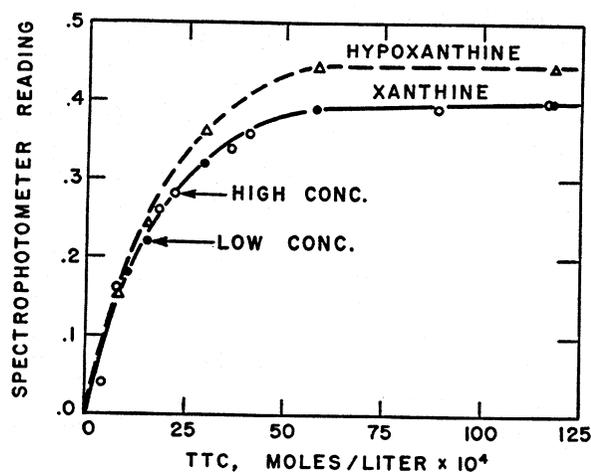


FIG. 2. Influence of triphenyl tetrazolium chloride (TTC) concentration on xanthine oxidase activity with hypoxanthine and two concentrations of xanthine. Concentrations in reaction mixture: xanthine 0.00182 (high) and 0.000182 (low) mole per liter; hypoxanthine 0.000059 mole per liter.

of TTC reduced is proportional to the time the enzyme acts on the TTC. The amount of TTC reduced is also proportional to the amount of enzyme. In routine assays two amounts of enzyme are used: 0.5 and 1.0 ml. of a suitable dilution as indicated in the procedure. This serves to establish the reproducibility of the assay. Also, if the 0.5-ml. value is lower than the 1.0-ml. value, the bubbling is likely to have been inadequate. If the 1.0-ml. value is low, the amount of enzyme used is too great (absorbance greater than 0.6). This should not occur if the dilutions of milk and cream indicated are used. Routine assays with this method are reproducible with less than  $\pm 5\%$  deviation.

Some work was also done with hypoxanthine instead of xanthine, and neotetrazolium instead of TTC. The reaction rate with hypoxanthine was about 25% faster than with xanthine, as was expected from previous observations (2, 5). Also, maximum reactivity occurred with more dilute solutions, as little as 0.00006 mole per liter being adequate (Figure 1). This difference between the two substrates has been observed recently with a purified xanthine oxidase (5). Neotetrazolium was found to react with the xanthine oxidase of milk at about the same rate as TTC. This substitute reagent was tried since it had been reported to have more than ten times the reactivity of TTC with insect xanthine oxidase (1), and it was hoped that its faster reaction might not have been inhibited by oxygen. It was found, however, that oxygen was inhibitory with it also.

*Application of the procedure.* The xanthine oxidase in raw market milk, as determined by these procedures, was found to be 140 units per milliliter for cream (separated by gravity, about 25% butterfat) and 25 units per milliliter for skimmilk. Cream from another source (25% butterfat) contained 210 units per milliliter.

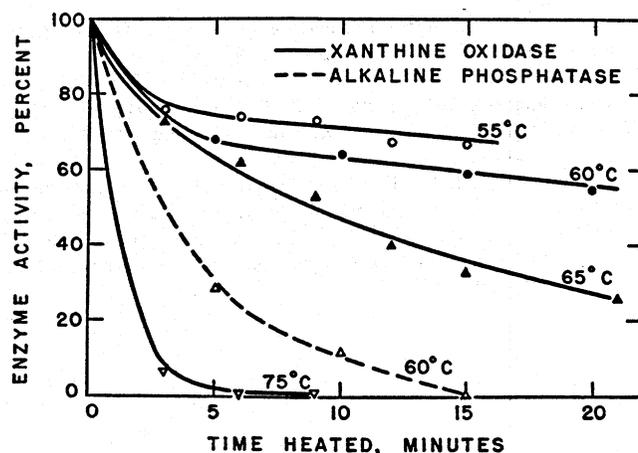


FIG. 3. Effect of heat on the xanthine oxidase and alkaline phosphatase in skim milk. Alkaline phosphatase determined with phenyl phosphate as substrate in ethanolamine buffer at pH 9.6 (7). The minutes heated do not include the time to come to temperature, which was 2 to 3 minutes.

The xanthine oxidase method has also been useful in characterizing the fat globule membrane and a related fraction in whey (8).

Another application of the method is illustrated in Figure 3, which compares the decrease in the xanthine oxidase, and also in the alkaline phosphatase activity, of skim milk when exposed to various elevated temperatures from 55 to 75° C. The decrease in alkaline phosphatase follows the usual pattern (3), and the xanthine oxidase, although more resistant to heat than alkaline phosphatase, decreases similarly.

#### SUMMARY

A method is described for the determination of xanthine oxidase in milk with triphenyl tetrazolium chloride. The enzymatic reaction is performed with nitrogen gas bubbling through the mixture to avoid oxygen which is inhibitory. Subsequent steps, which include extraction of the color with toluene after acidification with acetic acid, are performed in air since the red, reduced form of triphenyl tetrazolium chloride is stable. The effects of concentration of substrate and triphenyl tetrazolium, and other factors are investigated. The method is used to show the destruction of xanthine oxidase in skim milk at 55 to 75° C.

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