Studies on Estimation of Catalase Activity by the Use of Titanium Sulfate

SHIGERU FUJIMOTO
(The First Surgical Department, School of Medicine, Chiba University, Chiba, Japan)

SUMMARY
An improved method for the determination of catalase activity is described; it is a modification of the method of Patti and Bonet-Maury (21). The new method depends on the colorimetric determination of the yellow complex formed by titanium sulfate and H₂O₂, and eliminates some of the complications of the titration methods previously used.

MATERIALS AND METHODS

Materials.—The reagents used were reagent grade, except for titanium sulfate (Kokusan Chemical Works, Ltd.); de-ionized water was used throughout this experiment.

Stability of titanium sulfate solution.—The following concentrations of titanium sulfate were prepared by dissolving this compound in 2.5 N H₂SO₄: 0.01, 0.1, 0.5, 1.0, 3.0, 5.0, 10.0, 20.0, and 30.0 %. The optical density was measured at 1, 3, 6, and 24 hr, 10 and 30 days after preparing the solutions.

The color stability of the solutions was confirmed at 410, 415, and 420 μm periodically for a month, except for concentrations of titanium sulfate greater than 10 %.

The influence of titanium sulfate concentration on color development of the yellow complex, and determination of its optimal wavelength.—Four milliliters of each of the above-mentioned titanium sulfate solutions was added to 1.0 ml of 0.00667 M phosphate buffer (pH 6.8) containing 0.12 mg/ml H₂O₂; then optical density was measured at 410, 415, and 420 μm with the Hitachi spectrophotometer EPU-2A. Chart 1 shows the alteration of the optical density at 410, 415, and 420 μm, depending on the concentration of titanium sulfate.

The absorption spectrum of the yellow complex was determined, as shown in Chart 2. The maximum absorption was shown at 410 μm.

The stability of the yellow complex.—At intervals, the change of optical density of the yellow complex was examined. Samples were mixtures of 1.0 % titanium sulfate and H₂O₂ solutions with various concentrations, i.e., 0.04, 0.08, 0.12, 0.16, and 0.20 mg/ml.

Chart 3 shows changes of optical density when the yellow complex is allowed to stand at room temperature (18°C). It is clear from the curves shown in Chart 3 that optical density decreases rapidly within about 3 min and reaches a

1 Commercial titanium sulfate contains the insoluble substances present in H₂SO₄. A solution of 30.0 % titanium sulfate and H₂SO₄ was shaken vigorously, then centrifuged at 3000 rpm for 10 min. The supernatant of this solution was employed as 30 % titanium sulfate solution throughout this paper and was diluted as needed.
Chart 1.—Changes of optical density of the yellow complex depending on various concentrations of titanium sulfate. •—•, 410 m\(\mu\); ▲—▲, 415 m\(\mu\); ■—■, 420 m\(\mu\).

Chart 2.—Changes of optical density of the yellow complex, and titanium sulfate. ○—○, 0.18 mg/ml \(\text{H}_2\text{O}_2\); △—△, 0.14 mg/ml \(\text{H}_2\text{O}_2\); □—□, 0.07 mg/ml \(\text{H}_2\text{O}_2\); ●—●, 1.0% titanium sulfate (4.0 ml) + phosphate buffer (1.0 ml).

Plateau thereafter. Optical density changes at 25°C and 37°C were the same as shown in Chart 3. On this basis, optical density was measured at 10 min thereafter. The change of optical density at 10 min was plotted, depending on the concentration of \(\text{H}_2\text{O}_2\) (Chart 4). A linear relationship holds from 0.04 to 0.20 mg/ml of \(\text{H}_2\text{O}_2\).

**Tissue Catalase**

**Stability of titanium sulfate solution on the addition of the tissue homogenate.**—This experiment was carried out as follows: each 4.0 ml of the solution containing 0.01, 0.1, 0.5, 1.0, 3.0, 5.0, 10.0, and 20.0% of titanium sulfate was added to 0.95 ml of the phosphate buffer and 0.05 ml of the adequately diluted human-gastric cancer-tissue homogenate. The alteration of optical density of these mixtures was measured at 410 m\(\mu\). Chart 5 shows the stability of optical density as time goes on, within 5-17 min in dilute solution. It is clear that optical density remains fixed at the 10-min point after 0.05 ml of the dilute tissue homogenate and 0.95 ml of the phosphate buffer solution are added to 4.0 ml of 1.0% titanium sulfate.

Human liver, gastric cancer, and lymph node homogenates were employed in the next experiment using 1.0% titanium sulfate. Chart 6 shows the changes in optical density of these mixtures and centrifuged supernatant when diluted with tissue homogenate. The optical density of the supernatant is stable.

**The stability of the yellow complex with the addition of tissue homogenate.**—Tissue homogenate, 0.05 ml, was added to the yellow-complex solution, which made by reacting 0.95 ml of various \(\text{H}_2\text{O}_2\) solutions in Chart 3 and 4.0 ml of 1.0% titanium sulfate. As shown in Chart 7,
4. Each tube is placed in a water bath (5°C in this experiment) for a proper period of time (usually 1, 2, 3, 5, and 7 min).
5. Thereafter, 4.0 ml of 1.0% titanium sulfate in 2.5 N H$_2$SO$_4$ is added, and mixed vigorously.
6. About 10 min after the mixing, the optical density is determined at 410 nm, after centrifugation, if it is employed, for 5 min.
7. H$_2$O$_2$ consumed is calculated by the calibration curve as shown in Chart 4. The catalase activity is represented by the $K$ value found by extrapolation either from each optical density or from the amount of H$_2$O$_2$ consumed.

the optical density of the supernatant of these solutions was arranged almost on a straight line; the same results were obtained with the solutions of Chart 4.

As a result of experiments mentioned above, it was decided to measure catalase activities of tissue in the following manner.

Reagents:
1. 0.20 mg/ml H$_2$O$_2$ in 0.00667 M phosphate buffer, pH 6.8;
2. 0.00667 M phosphate buffer, pH 6.8;
3. 1.0% titanium sulfate in 2.5 N H$_2$SO$_4$;
4. Properly diluted catalase solution.

Procedure:
1. Tissue homogenate is diluted properly with phosphate buffer.
2. Five-hundredths milliliter of the diluted tissue homogenate is placed into each of 6 test-tubes.
3. Phosphate buffer, 0.95 ml, is added to 1 test-tube (the blank); to each of the others is added 0.95 ml of 0.20 mg/ml H$_2$O$_2$ in 0.00667 M phosphate buffer. These are mixed by shaking gently.
Reproducibility and proportionality between activity and the amount of tissue homogenate.—Chart 8 presents data to indicate that, by the procedure mentioned previously, good reproducibility and proportionality between optical density and the amount of liver homogenate can be obtained within proper limits of homogenate concentration.

Relationship between color development and time of incubation.—Linearity of optical density with time of incubation was ascertained in various concentrations of liver homogenate. Chart 9 shows linearity between the two in 3 min.

According to the procedure described above, first-order kinetics are being followed for about 3 min, as revealed in Charts 8 and 9. In the first-order kinetics, the following formula is given.

\[ K = \frac{1}{t} \log \frac{a}{a - x} \]

where \( K \) is the velocity constant, \( a \) is the amount of \( \text{H}_2\text{O}_2 \) at zero time, \( a - x \) is the amount of \( \text{H}_2\text{O}_2 \) at 1, 2, 3, 5, or 7 min, and \( t \) is time in min.

From the open and solid circles,

\[ \log \frac{a}{a - x} \]

at each time on Chart 9 was estimated; this was plotted at each time interval on Chart 10. As far as these plots remain straight lines, first-order kinetics are followed. On the right-hand ordinate, \( K \), the velocity constant, was calibrated, and the points of intersection with the lines were taken. Both \( K \) values agreed. On the left-hand ordinate the amounts of consumed \( \text{H}_2\text{O}_2 \) were recorded. The dash lines indicate the amounts consumed at each point of time. Beyond the range, where the first-order kinetics were not followed, the theoretic values of \( \text{H}_2\text{O}_2 \) expected to be consumed were calculated; the open and solid squares were plotted on the dotted lines. As shown on these charts, the first-order kinetics occur for 3 min. It may be attributable to the denaturation of the enzyme that after this time, first-order kinetics are not followed.

Comparison with the results obtained by various methods.—Table 1 shows a comparison of the results obtained by this titanium method and by the methods of Beers and Sizer and Euler and Josephson. There are only small differences among the 3 methods.

**DISCUSSION**

Since the report of the reaction of titanium sulfate with \( \text{H}_2\text{O}_2 \) by Schönn (25) in 1870, this reaction has been utilized especially for qualitative analysis of titanium. Backer and Goward (3) mentioned that the so-called yellow complex can be detected as long as 1 gm of zirconium contains 10–100 ppm of titanium. According to Lewis (16), and Babko and Volkova (2), \( 10^{-4} \) m titanium concentration is
enough to develop the color of the yellow complex and the maximum absorption occurs at 405-410 m\(\mu\).

Neal (20), and Backer and Goward (3) also pointed out that maximum absorption occurs at 410 m\(\mu\) and that the product is stable for at least 24 hr. This reaction was also studied by Garrigues (11), Viallet (29), and Ciavatta and Liberti (9).

The author has applied this titanium reaction to the estimation of catalase activity, modifying the method of Patti and Bonet-Maury (21). At present, the most widely used methods for measuring catalase activity are those of Euler and Josephson (10) and Sumner (28); these methods may be summarized as follows. The reaction of catalase is stopped with H\(_2\)SO\(_4\) and the remaining substrate is tritiated with KMnO\(_4\) or thiosulfate. On the other hand, titanium sulfate dissolves only in H\(_2\)SO\(_4\) or HCl (19) and produces a yellow complex by reacting with H\(_2\)O\(_2\). When the concentration of titanium sulfate is constant and low, this yellow color is proportional to the concentration of H\(_2\)O\(_2\). If this reaction is applicable to the determination of catalase activity, we might have 2 advantages: the simultaneous interruption of the reaction and the color development. However, Patti and Bonet-Maury divided this process into 2 steps. Interruption of the catalase reaction was caused with 4 drops of H\(_2\)SO\(_4\); thereafter, the color was developed with 2 drops of titanium sulfate solution.

Titanium sulfate (TiOSO\(_4\)) is formed by the combination of TiOSO\(_4\) and TiO\(_2\) (18, 19). The reaction for the production of the yellow complex may be shown as follows (2, 18, 19, 21, 23, 24).

\[
\text{TiOSO}_4 + \text{H}_2\text{O} + \text{H}_2\text{SO}_4 \rightarrow \text{H}_2\text{[TiO}_2\text{(SO}_4)_2\text{]} + \text{H}_2\text{O}
\]

This reaction of titanium sulfate is inhibited with F\(^-\), I\(^-\), Cl\(^-\), Br\(^-\), NO\(_3\)^- and CH\(_3\)COO\(^-\) (8, 23, 24). On the basis of this, 0.00667 m phosphate buffer, pH 6.8, which is utilized in the Euler-Josephson method (10) was employed; 1.0% titanium sulfate in 2.5 n H\(_2\)SO\(_4\) was used to stop the reaction and develop the color.

Euler and Josephson (10) adopted 2.0 n H\(_2\)SO\(_4\) to stop the reaction in the measurement of catalase activity. According to Backer and Goward (3), titanium sulfate dissolved in 1.5-3.5 n H\(_2\)SO\(_4\). But 2.5 n H\(_2\)SO\(_4\) gave the most satisfactory results for the measurement of catalase activity in this experiment.

Lewis (16), Neal (20), and Backer and Goward (3) studied the solution at 405-410 m\(\mu\), and Patti and Bonet-Maury (21) adopted 450 m\(\mu\), while this author proposed 410 m\(\mu\), because of the stability of the optical density regardless of the presence of tissue homogenate along with the yellow complex.

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