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# Quantitative Measurement of Bacterial Growth by the Reduction of Tetrazolium Salts

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Tetrazolium salts can be used for measuring the reducing activity of microbial cultures colorimetrically, because the reduced form of these redox indicators is conspicuously colored (B. Jámbor, *Tetrazolium Salze in der Biologie*, G. Fischer, Jena, 1960; B. J. Liska et al., J. Dairy Sci. **41**:776, 1958).

In an actively growing culture, the reducing activity is proportional with the number of bacteria in the culture, assuming that all bacteria are in a similar metabolic state. We correlated the reducing activity of nutrient broth cultures of *Escherichia coli* and *Staphylococcus aureus* with their viable count. We have found that under controlled conditions the color intensity of the reduced tetrazolium salt, formed by the reducing activity of bacteria, was proportional with the number of actively growing bacteria in the culture. Although tetrazolium salts inhibit the growth of bacteria to some extent (E. D. Weinberg, J. Bacteriol. **66**:240, 1953; P. S. May et al., Proc. Soc. Exptl. Biol. Med. **105**:364, 1960), we used tetrazolium salts in a concentration that had very little growth inhibition, or we used them under conditions which did not affect the quantitative determination of bacterial growth.

Triphenyl tetrazolium chloride (TTC; Nutritional Biochemicals Corp., Cleveland, Ohio) and blue tetrazolium (BT; DAJAC Labs, Philadelphia, Pa.) were used in the experiments. The growth inhibitory effect of TTC and BT for the two test organisms is shown in Table 1. Since BT was more toxic than TTC for these bacteria, we carried out most experiments with TTC, although similar results were also obtained with BT. The TTC concentration used in the tests was 0.1 the 50% inhibition concentration. The TTC reduction of *E. coli* and *S. aureus* cultures during a 30-hr incubation period with TTC is shown in Fig. 1. The red formazan (the reduced form of TTC) was extracted from the culture broth with an equal amount of *n*-butanol, according to Barnes (E. M. Barnes, J. Gen. Microbiol. **14**:57, 1956). The *n*-butanol-culture broth mixture was kept frozen overnight before the two layers were separated by centrifugation. The color intensity of the clear upper layer (the *n*-butanol solution of formazan) was measured in a Spectronic-20 colorimeter at 490 mμ. An *n*-butanol extract of a control culture was used for a blank.

The TTC reducing activity of the culture was proportional with the number of newly formed cells, i.e., the actively growing portion of the bacterial population. This point is clear especially in Fig. 2. The TTC reduction was carried out with different inoculum levels. The log $A/A_0$ ratio represents the increment in the population of the growing culture. The increase in optical density was approximately the same for an equal increment in bacterial growth, independently from the level of inoculum. The color intensity of the culture at the time of inoculation of the nutrient broth was obtained by also adding the same amount of inoculum to 10 ml of buffered saline (*p*H 7.2) containing 0.01 mg of TTC, and measuring the color intensity after 24 hr of incubation. The differences between the TTC reduction of a $1.0 \times 10^4$ and a $1.0 \times 10^7$ bacteria/ml washed cell suspension (stationary culture) were very small

TABLE 1. *Inhibiting effect of tetrazolium salts on the viable count of bacteria*

| Microorganism | Concn (mg/ml) causing 50% growth inhibition | |
|---|---|---|
| | BT | TTC |
| *Escherichia coli* | 0.029 (±0.002) | 0.09 (±0.001) |
| *Staphylococcus aureus* | 0.001 (±0.002) | 0.10 (±0.002) |

[a] BT and TTC concentrations are given in the final bacterial growth medium. Figures in parentheses represent the standard deviation, determined from 10 identical tests. Inhibition was measured by inoculating a suspension of $1.0 \times 10^5$ bacteria/ml, thrice-washed in buffered saline (*p*H 7.2), into 10 ml of nutrient broth containing increasing amounts of TTC or BT, and then making plate counts of the bacteria after 24 hr of incubation at 37 C.

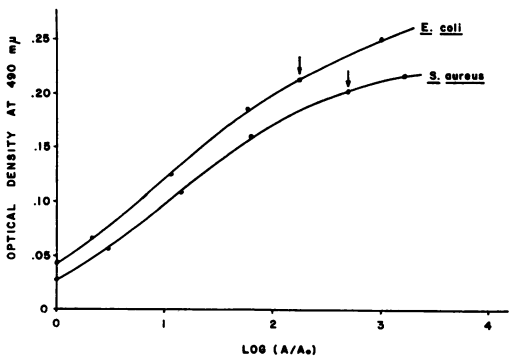


FIG. 1. *TTC reduction as the function of the number of viable bacteria. A = the number of bacteria in the culture at a given time during the 30-hr incubation period with TTC (by plate count); $A_0$ = the number of bacteria inoculated into nutrient broth. Procedure: 10 ml of nutrient broth was inoculated with a suspension of 1.0 × $10^5$ bacteria/ml, thrice-washed in buffered saline (pH 7.2), and 0.1 ml of aqueous solution of TTC (0.01 mg/ml) incubated at 37 C in the presence of air for 30 hr. Samples for plate count and for colorimetry were taken every 6 hr. The arrows indicate the bacterial increment at the 24th hr. The average error of the colorimetric determination was 7.5%, by use of the plate count values for reference (determined from five independent assays).*

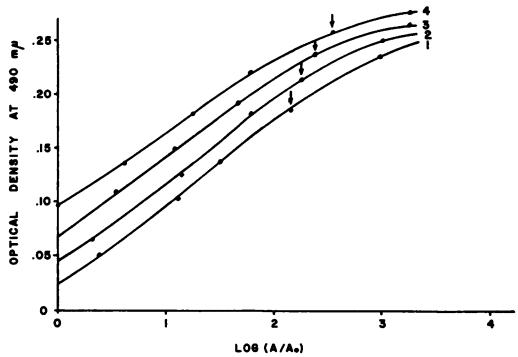


FIG. 2. *Effect of the inoculum level on the TTC reduction of Escherichia coli. Conditions were the same as in Fig. 1, except the amount of inoculum was (1) 5.0 × $10^4$, (2) 1.0 × $10^5$, (3) 1.0 × $10^6$, and (4) 1.0 × $10^7$ bacteria /ml. The arrows indicate the number of bacterial increment at the 24th hr.*

as compared to the growing culture (Fig. 2). This indicates strongly that only growing bacteria can reduce TTC.

In a log-phase culture, 3 to 4 hr of incubation with TTC is adequate for developing a measurable color intensity, although in the experiments presented in Fig. 1 and 2 the TTC was present from the time of inoculation. For determining the actively growing portion of a bacterial population, it would be adequate to add TTC to samples of the growing culture at selected intervals and to determine the color intensity after 3 to 4 hr of incubation. Since the color intensity is proportional with the number of bacteria present before TTC addition, the number of actively growing bacteria at the time of TTC addition can be calculated from calibration curves, such as those shown in Fig. 2, prepared with known numbers of the bacterium.

Since the respiratory activity of a growing microbial culture is associated with a decreasing redox potential (L. F. Hewitt, *Oxidation Reduction Potentials in Bacteriology and Biochemistry*, E. & S. Livingstone, Edinburgh, 1950; R. P. Tengerdy, J. Biochem. Microbiol. Technol. Eng. 3:241, 1961) and since the TTC reduction test measures the number of cells that have a low redox potential, this test could be used especially advantageously for studying quantitatively the effect of respiratory inhibitors on the growth of bacteria.

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