

Precise Adsorption Method for Measuring the Percentage of Dead Bacterial Cells

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ABSTRACT

VAIRO, MARINA L. R. (University of São Paulo, São Paulo, Brazil) AND WALTER BORZANI. Precise adsorption method for measuring the percentage of dead bacterial cells. *Appl. Microbiol.* **10**: 500–503. 1962.—It was observed that Freundlich's law is obeyed when methylene blue is adsorbed from different aqueous solutions by mixtures of dead and live bacterial cells. A simple and precise colorimetric method was developed for determining the percentage of dead cells.

Borzani and Vairo (1960a) proposed a method for the evaluation of the percentage of dead cells in suspensions of *Sarcina lutea*, based on the fact that, when this percentage is greater than about 40%, the adsorption of methylene blue by the dead cells follows Freundlich's law. It was also observed (Borzani and Vairo, 1960a) that the described method was not applicable to *Bacillus subtilis*, *Serratia marcescens*, and *Escherichia coli*, because dead and live cells of these microorganisms adsorb practically the same quantity of methylene blue.

The purpose of this paper is to show that (i) the convenient choice of the experimental conditions leads to the applicability of Freundlich's law to the adsorption of dye by mixtures of dead and live cells of bacteria other than *S. lutea*, (ii) the applicability of Freundlich's law is observed when the percentage of dead cells varies from 0 to 100%, and (iii) the simple and precise method for the measurement of the percentage of dead yeast cells, proposed in a previous paper (Vairo, 1962b), can be applied to some bacteria.

This report shows that the adsorption of certain dyes by mixtures of dead and live bacterial cells follows Freundlich's law when the percentage of dead cells varies from 0 to 100%, and describes a method for the measurement of the percentage of dead bacterial cells.

Application of Freundlich's law. The physicochemical law of Freundlich, already mentioned in other papers (Borzani and Vairo, 1958, 1959, 1960b; Vairo, 1962a, 1962b), can be written:

$$\frac{C_i - C_f}{C_f^n} = KPC$$

where C_i is the initial dye concentration, C_f is the concentration at the equilibrium point, C is the cell concentration, and P is the percentage of dead cells; K and n are constants that depend upon the experimental conditions.

In this particular case, C is constant and P varies from 0 to 100%. The previous equation, then, can be written:

$$\frac{C_i - C_f}{C_f^n} = K'P$$

MATERIALS AND METHODS

S. lutea, *B. subtilis*, *S. marcescens*, and *E. coli*, grown on glucose agar (Salle, 1954), were used in the experiments.

Two stains were successfully tested. Stain 1, Fink and Kühles' methylene blue (Joergensen, 1948), consisted of: methylene blue, 200 mg per liter; KH_2PO_4 , 27.2 g per liter; and Na_2HPO_4 , 71 mg per liter. Stain 2 consisted of methylene blue, 100 mg per liter; Na_2HPO_4 , 14.6 g per liter; and citric acid, 10.2 g per liter. The pH varied from 4.8 to 5.0.

Total cell concentration was measured in grams of dry matter per liter of suspension (White, 1954).

Stock bacterial suspensions were prepared as follows. A known mass of bacteria was mixed with distilled water and agitated for 20 min to disperse the aggregated cells; when a dead cell suspension was desired, the cells were killed by boiling for 30 min. The suspensions were then diluted with distilled water to the desired volume to give a known total-cell concentration.

From the stock suspensions of dead or live cells, suitable volumes were pipetted and mixed together in 50-ml volumetric flasks to prepare suspensions of equal total bacterial concentration but different dead-cell percentage. A 25-ml amount of stain 1 was then added, and the volumes were diluted with distilled water to the desired level (10 ml of stain 2 were used in the experiments with *S. marcescens*). The mixtures were agitated at room temperature for 20 min and then centrifuged at 2,000 to 2,500 $\times g$ (1,400 to 1,600 $\times g$ were sufficient for *S. lutea*) for 20 to 30 min to separate the cells.

The methylene blue concentrations were measured colorimetrically (Coleman Junior spectrophotometer) at 440 m μ .

Only typical results are presented in this report.

RESULTS

Figure 1 shows that the adsorption of stain 2 by *S. marcescens* obeys Freundlich's law only when the percentage of dead cells is greater than about 30%. Similar

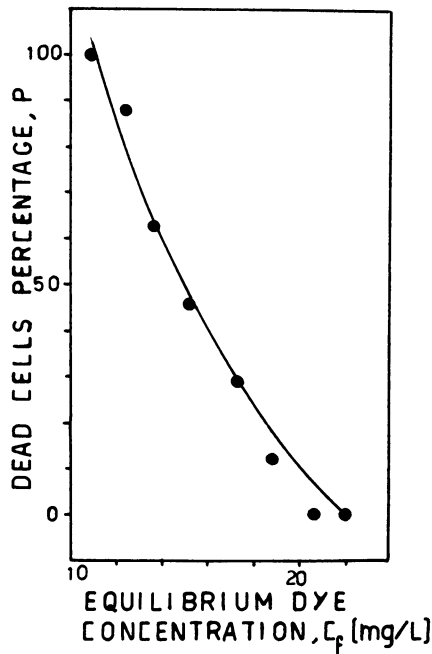


FIG. 1. Relationship between the percentage of dead cells and the equilibrium dye concentration. Experiments carried out with *Serratia marcescens* and stain 2. Initial dye concentration = 22.0 mg per liter; cell concentration = 0.261 g per liter. Equation: $(22.0 - C_f)/C_f^{0.91} = 0.0121 P$.

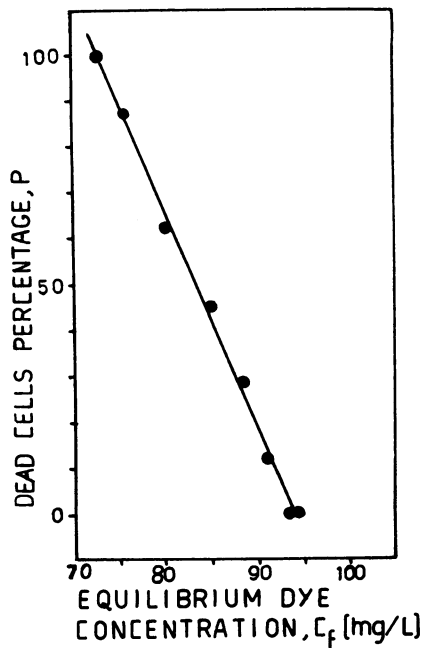


FIG. 2. Relationship between the percentage of dead cells and the equilibrium dye concentration. Experiments carried out with *Sarcina lutea* and stain 1. Initial dye concentration = 94.4 mg per liter; cell concentration = 0.386 g per liter. Equation: $P = 442.9 - 4.71 C_f$.

results were already obtained with *S. lutea* and methylene blue solutions (Borzani and Vairo, 1960a).

Figures 2, 3, and 4 show the results obtained when stain 1 was adsorbed by *S. lutea* and *E. coli*. The linear relations

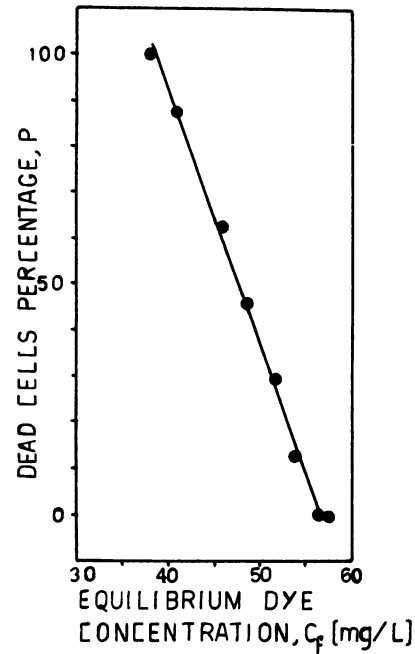


FIG. 3. Relationship between the percentage of dead cells and the equilibrium dye concentration. Experiments carried out with *Escherichia coli* and stain 1; initial dye concentration = 57.5 mg per liter; cell concentration = 0.270 g per liter. Equation: $P = 312.4 - 5.50 C_f$.

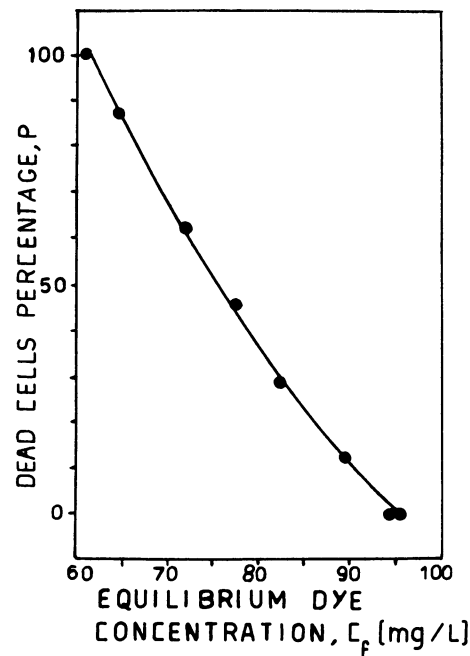


FIG. 4. Relationship between the percentage of dead cells and the equilibrium dye concentration. Experiments carried out with *Sarcina lutea* and stain 1; initial dye concentration = 95.5 mg per liter; cell concentration = 0.542 g per liter. Equation: $(95.5 - C_f)/C_f^{0.86} = 0.00973 P$.

presented in Fig. 2 and 3 are particular cases of Freundlich's law, already observed in a previous article (Borzani and Vairo, 1960b). Figures 2, 3, and 4 show that the adsorption law is obeyed when P varies from 0 to 100%.

Several tests were carried out with *B. subtilis* and different dyes. In all cases, the dead and live cells adsorbed practically the same quantity of dye.

Precise adsorption method. The linear relations observed between the percentage of dead cells and the dye concentration at the equilibrium (Fig. 2 and 3) permit the establishment of a simple and precise method for the evaluation

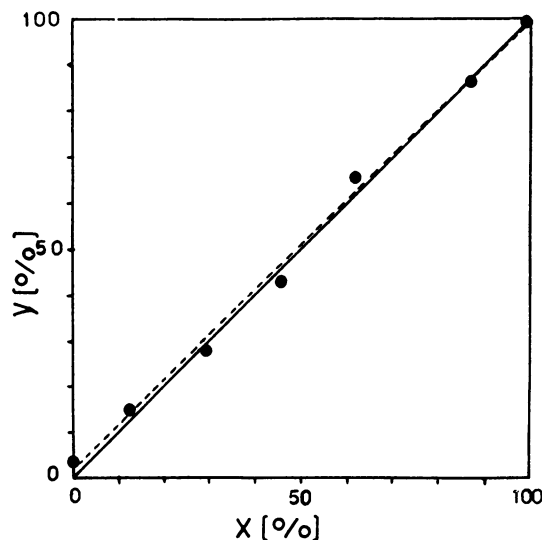


FIG. 5. Relationship between the percentage of dead cells of *Sarcina lutea* determined by the proposed method (Y) and the real percentage of dead cells (X). Experimental equation ($Y = 2.01 + 0.97 X$) and theoretical equation ($Y = X$) are represented.

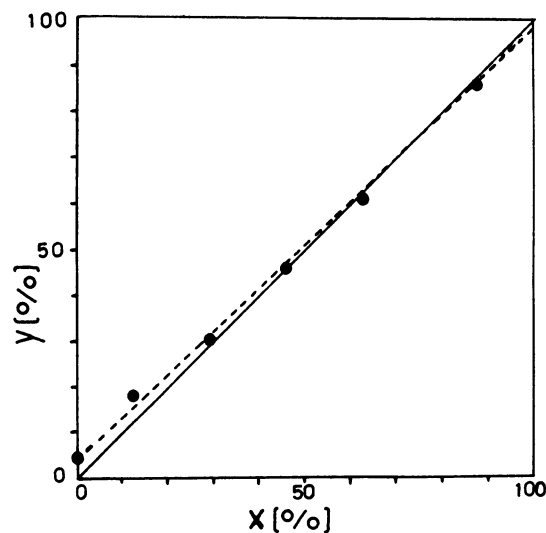


FIG. 6. Relation between the percentage of dead cells of *Escherichia coli* determined by the proposed method (Y) and the real percentage of dead cells (X). Experimental equation ($Y = 3.8 + 0.94 X$) and theoretical equation ($Y = X$) are represented.

of the percentage of dead bacterial cells, analogous to the method described for yeasts (Vairo, 1962b).

This method is as follows. The original bacterial suspension was divided into two equal volumes; one volume was boiled to kill the cells and then cooled. Stain 1 was added to each suspension, and the mixtures were diluted with distilled water to give a total cell concentration of about 0.4 g per liter for *S. lutea*, about 0.3 g per liter for *E. coli*, and methylene blue concentrations of about 100 and 60 mg per liter for *S. lutea* and *E. coli*, respectively. The mixtures were agitated at room temperature for 20 min, centrifuged ($1,400 \times g$ for *S. lutea* and $2,000 \times g$ for *E. coli*) for 20 to 30 min to separate the cells, and the final dye concentrations were measured. With C_i as the initial dye concentration and $C_{f(100)}$ as the equilibrium dye concentration when the percentage of dead cells is 100%, the following equation was established.

$$P = 100 \cdot \frac{C_i - C_f}{C_i - C_{f(100)}}$$

The percentage (P) of dead cells in the initial bacterial suspension can be calculated, since the equilibrium dye concentration (C_f) was also determined.

Figures 5 and 6 show the results obtained in typical experiments carried out to test the described colorimetric method.

DISCUSSION

This paper demonstrates that the choice of suitable experimental conditions, particularly those concerning the composition of the dye solution, profoundly affects the dye adsorption by dead bacterial cells. Comparison of previous results (Borzani and Vairo, 1960a) with our results distinctly clarifies the above statement.

Thus, it seems logical to suppose that a convenient choice of the composition of the dye solution will permit the application of Freundlich's law to the adsorption of dye by mixtures of dead and live cells of any bacteria.

The method described in this article presents the same advantages of the analogous method presented for yeasts (Vairo, 1962b).

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LITERATURE CITED

- BORZANI, W., AND M. L. R. VAIRO. 1958. Quantitative adsorption of methylene blue by dead yeast cells. *J. Bacteriol.* **76**:251-255.
- BORZANI, W., AND M. L. R. VAIRO. 1959. Quantitative adsorption

- of crystal violet by dead microorganisms. *J. Bacteriol.* **77**: 757-759.
- BORZANI, W., AND M. L. R. VAIRO. 1960a. Adsorption method for determining the percentage of dead cells in suspensions of *Sarcina lutea*. *J. Bacteriol.* **80**:574.
- BORZANI, W., AND M. L. R. VAIRO. 1960b. Adsorption of methylene blue as a means of determining cell concentration of dead bacteria in suspensions. *Stain Technol.* **35**:77-80.
- JOERGENSEN, A. 1948. *Microorganisms and fermentation*, p. 223. Charles Griffin and Co. Ltd., London.
- SALLE, A. J. 1954. *Laboratory manual on fundamental principles of bacteriology*, 4th ed. McGraw-Hill Book Co., New York.
- VAIRO, M. L. R. 1962a. Influence of several factors on the applicability of Freundlich's law to the adsorption of methylene blue by dead yeast cells. *Appl. Microbiol.* **10**:141-145.
- VAIRO, M. L. R. 1962b. A modified adsorption method for determining percentage of dead yeast cells. *Biotechnol. Bioeng* **4**:247-254.
- WHITE, J. 1954. *Yeast technology*, p. 144. Chapman and Hall, London.