NOTES

Gradient Technique to Test the Effects of Substances on Fluorescent Antibody Reactions

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A simple technique is described for making gradients of substances, applying them to bacterial cells during fluorescent antibody reactions, and observing their effects.

In the course of developing a fluorescent antibody (FA) reaction, it was desirable to test the effects of certain ions on the combination of antibody with bacterial cells (4). Preparation of substances at multiple concentration levels, pH adjustments, and handling of multiple microcopy slides are very time-consuming; therefore, techniques were developed to simplify these processes. The following communication describes methods for preparing gradients of test substances (in the presence of the FA reactants), applying them to bacterial cells, and making uninterrupted microscopy observations of their effects. Also described is a method for breaking the gradient into discrete fractions which can be applied to bacteria on individual glass slides.

The device shown in Fig. 1a is used to produce the gradients. The reactants for the FA test, such as antibody and saline, are added in equal concentrations to both chambers of the device, except that chamber B also contains the substance to be tested, plus 20% sucrose. The tubing connecting the two chambers should first be filled with the solution for chamber A and clamped off. Then 2.0 ml of the proper solution is placed in each chamber. After starting the stirring motor and removing the forceps separating the two solutions, the forming gradient is allowed to flow into a long glass tube (Fig. 1b), while the flow rate is controlled at about 1.0 ml per min by squeezing the delivery tube with a hand-held hemostat. The glass strip shown in the tube is coated with dried, fixed bacterial cells. As the gradient fills the glass tube, the polyethylene tubing is gradually lifted upward so that the effluent end is kept just above the meniscus of the forming gradient. The result is a gradient of the test substance within a gradient of sucrose in the presence of a constant antibody concentration. The sucrose gradient, which is most dense at the bottom and least dense at the top, prevents migration of ions. This was tested by forming a pH gradient mixed with bromothymol blue indicator. The color gradient of the indicator remained stable in excess of 18 h.

The gradient is allowed to remain in contact with bacteria on the glass strip until complete antigen-antibody coupling has occurred (2 h at 37°C was found adequate with the system tested; at the end of this time antigen-antibody reactions under high and low sucrose concentrations were equal). To remove the gradient, about 2.0 ml of chilled buffer is layered above it, the rubber ampoule stopper is removed, the contents are allowed to drain out rapidly, and the tube immediately is refilled with chilled buffer. This step is important in preventing zones in the gradient from smearing across and reacting with bacteria in adjacent zones. Additional washing is done for 10 to 20 min inside the tube.

To evaluate the effects of the gradient, the treated glass strip is cover-slipped by using strips cut from 60-mm-cover glass stock with a diamond-tipped pencil, and scanned along its length under dark-field fluorescence microscopy. In order to aid in scanning, the strip may be attached with contact cement along one edge of a thin template which has been cut to fit in the stage slide holder. The stop on the stage vernier mechanism may also be removed to provide a wider movement across the field of view. While scanning along the strip, visual
estimation of changes in fluorescence intensity allows continuous comparison of any one area with that area just previously observed. By correlating fluorescence intensity with distance traversed, one can determine the concentration of a test substance in contact with cells at that point. This is aided by use of a nomograph, as described in the third paragraph below.

If it is desirable to prepare multiple solutions of different concentrations for use on individual microscope slides, the sucrose may be deleted and the device used as shown in Fig. 1c. While the gradient forms, discrete fractions are collected in sections of constant-bore disposable pipette tubing cut to contain exactly 0.1 ml as the section is held upright on strips of laboratory tape.

By using the setup shown in Fig. 1c, it was possible to test the accuracy of the device in producing linear gradients. A hydrogen-ion gradient was produced by the device and collected in discrete fractions. Each fraction was measured for pH with a miniature electrode on an expanded scale meter, and the values were compared with predicted values for perfect linear mixing. The upper two curves in Fig. 2 show that the device produces nearly perfect linear gradients. When sucrose and serum proteins are added, the pH of the fractions shift (lower curve of Fig. 2). The displacement of the curve is caused by viscosity of the sucrose solution contained in chamber B, because addi-

tion of serum proteins alone produces a curve similar to the measured buffer curve. The shoulder in the curve between pH 7.4 and 7.55 is reproducible and probably occurs because the sucrose is diluted out past a point of critical viscosity.

The nomograph shown in Fig. 3 was used to determine the concentrations of calcium and magnesium ion gradients along glass strips used in FA reactions. The nomograph was constructed by plotting the degree of deviation of points along the lower curve in Fig. 2 from the equivalent theoretical points of the upper curve in Fig. 2, and then substituting molarity values for pH values.

The gradient device and techniques described in this paper provide a semiquantitative, but rapid, method for studying the effects of any compound on the kinetics of the fluorescent antibody reaction over a wide concentration range. The relative accuracy of the technique is shown in a separate communication; the results compare favorably with those obtained from microphotometric measurements of fluorescent cells treated with discrete fractions over a pH range (4). The pH of optimal antibody coupling found by the more rapid gradient technique differed by only 0.05 units from that found by precise photometric measurement of individual fractions.

![Diagrammatic sketch of apparatus used for the formation and collection of gradients.](image)

**Fig. 1.** Diagrammatic sketch of apparatus used for the formation and collection of gradients.

![Accuracy of the gradient device in forming linear gradients. The measured pH values of discrete 0.1-ml fractions of buffer drawn from the gradient device were based upon a starting pH of 5.7 phosphate-buffered saline (PBS) in chamber A and pH 8.0 PBS in chamber B. Theoretical points were found by calculating 200 discrete theoretical molar concentrations followed by extrapolation of pH from data given by Gomori (5); every fifth concentration is plotted in the figure. The pH values shown for fractions containing buffer, sucrose, and serum proteins are based upon the same starting pH values as for the other two curves; however, the solutions contained 1% bovine serum albumin fraction V, antiserum diluted 1:20, and a starting concentration of 20% sucrose in chamber B.](image)

**Fig. 2.** Accuracy of the gradient device in forming linear gradients. The measured pH values of discrete 0.1-ml fractions of buffer drawn from the gradient device were based upon a starting pH of 5.7 phosphate-buffered saline (PBS) in chamber A and pH 8.0 PBS in chamber B. Theoretical points were found by calculating 200 discrete theoretical molar concentrations followed by extrapolation of pH from data given by Gomori (5); every fifth concentration is plotted in the figure. The pH values shown for fractions containing buffer, sucrose, and serum proteins are based upon the same starting pH values as for the other two curves; however, the solutions contained 1% bovine serum albumin fraction V, antiserum diluted 1:20, and a starting concentration of 20% sucrose in chamber B.
The device for forming the gradients is not unique, because it could be substituted by any commercially available gradient former which can handle small volumes. The device is a smaller-scale version of a design described by Bock and Ling (1) and adapted by others (2, 5). These latter devices were developed for gradient elution chromatography or density gradient centrifugation. Unique to the apparatus described in this paper is its use in producing a stabilized gradient of ions against a strip of cells in the presence of reacting FA reagents. However, if another device is used, its flow characteristics should be checked by some method, such as the use of a refractometer (5) or comparison of a pH profile with the theoretical pH, as was done in the present case.

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