Different pH Optima in the Two Steps of an Indirect Fluorescent Antibody Reaction for Clostridium botulinum Type E

RICHARD A. ROBOHM
Middle Atlantic Coastal Fisheries Center, National Marine Fisheries Service, National Oceanic and Atmospheric Administration, Milford, Connecticut 06460

Received for publication 1 August 1973

Quantitative measurements of the effects of ions on fluorescent antibody reactions have not been reported in the literature. Data in this report show the effects of ranges of H+, phosphate, Mg2+, and Ca2+ molarities on antigen-antibody coupling during an indirect fluorescent antibody (IFA) reaction for Clostridium botulinum type E. These effects were quantified in two ways: (i) by microphotometric measurement of cell fluorescence intensity; and (ii) by visual estimation of cell fluorescence intensity on long glass strips treated with antibody in ion gradients. Optimal pH for the first part of the reaction (coupling of rabbit antibody to cells) was 7.25, and optimum in the second part (coupling of tracer globulin to the rabbit globulin) was pH 7.37. Running the reaction as little as 0.10 to 0.15 pH units off from the optima considerably reduced fluorescence intensity. Sodium phosphate buffer up to 0.1 M did not significantly affect either portion of the reaction. Ca2+ and Mg2+ showed no effect on the first part of the reaction. These results support the use of higher-strength phosphate buffer and indicate for the first time that IFA reactions may have two independent, narrow pH optima.

Most texts on fluorescent antibody (FA) techniques recommend a general-use buffer formula. Cherry et al. (1), Coons (2), Nairn (8), and Goldman (3) suggest holding dye-conjugated antibodies in saline buffered with 0.01 M phosphate. Kawamura (4) prefers 0.005 M phosphate. The pH value of these buffers is listed variously at pH 7.0 by Coons (2) and Kawamura (4), pH 7.1 by Nairn (8), pH 7.5 by Cherry et al. (1), and between pH 7.0 and 7.5 by Goldman (3). Although many investigators undoubtedly run their reactions at a pH based upon visual estimation of best fluorescence intensity, only one publication can be found describing the examination of pH effects during a fluorescent antibody reaction. Based upon visual observations of FA-stained Escherichia coli, I. F. Mikhailov (Fed. Proc. 25:14, 1966) reported that flakes of fluorescent conjugate precipitated out below pH 6.0 and poor staining occurred below pH 4.0, but he found no effect within the pH range of 6 to 10.

This communication describes the effects of altering pH and phosphate molarity during both portions of an indirect fluorescent antibody (IFA) reaction for Clostridium botulinum type E. Specifically, measurements were made on both the coupling of rabbit antibody to bacterial cells and the coupling of fluorescent tagged sheep anti-rabbit globulin to the rabbit antibody during changes in pH and phosphate molarity. Measurements were also made during the first part of the IFA reaction under varying concentrations of Mg2+ and Ca2+. The effects on the IFA reaction were quantified in two ways: (i) by microphotometric measurement of cell fluorescence intensity; and (ii) by visual estimation of cell fluorescence intensity on long glass strips treated with antibody in ion gradients (10).

Although microphotofluorimetry has been used in a variety of fluorescence applications, this is the first time it has been employed to measure quantitatively the effects of ions on antigen-antibody coupling during FA reactions. (This report is from part of a dissertation submitted to the Univ. of Michigan in partial fulfillment of requirements for the Ph.D degree.)

MATERIALS AND METHODS

Antiserum. Vegetative cells of C. botulinum type E (Kalamazoo isolate) were grown in the Trypticase-peptone-glucose (TPG) medium of Schmidt et al.

179
(12). After 55 h of growth at 20 °C, cells were chilled and washed three times in physiological saline buffered at pH 7.2 with 0.1 M phosphate (PBS). Cell walls were obtained in the following manner. A slurry of 1 part of cells in 8 parts of PBS was prepared, and 4.0-ml samples were added with 2.0 g of washed glass beads (0.18 mm; Glasperlen, B. Braun, Melsungen, Germany) to a stainless steel cup equipped with a cooling jacket. The cells were sonicated in the cup for 3 min by using a Bronwill Biosonic II disintegrator (Bronwill Scientific, Rochester, N.Y.) at maximal amplitude (2 kc/s at 125 W). Water at 0 °C was run continuously through the cooling jacket during sonic treatment. Ninety-nine percent of the cells were broken by this procedure. The fractured cells were washed twice in PBS. The cell walls were separated from spores, unbroken cells, and cell contents by passing them through a 40-ml continuous gradient of 40 to 4% sucrose in PBS, pH 7.2, at a centrifugal force of 640 × g for 30 min in a Sorvall HB-4 head. The upper of two dense bands formed in the gradient tube was removed with a 22-gauge needle and passed through a second sucrose density gradient, and the material was washed twice in PBS, pH 7.2. The washed pellet was resuspended in buffered saline to a reading of 142 on a Klett-Summner colorimeter (420-nm filter). The resuspended material was confirmed to be broken cell walls by phase contrast observations and bright-field microscopy of prepara-
tions layered with a flagella stain. The suspensions were quick-frozen in an alcohol-dry ice mixture and stored at −25 °C.

New Zealand white rabbits were immunized by alternating intraperitoneal (ip) and intravenous (iv) injections of cell-wall suspension every 7 days for 4 weeks (0.35, 0.5–1.1, and 1.4-ml doses were given consecutively). The ip injections were made in the presence of an equal volume of Freund complete adjuvant. After a 2-month resting period, the rabbits were given 0.7-ml doses iv and ip on consecutive days and restrained via the central ear artery 7 days later. Serum was separated, 1:10,000 merthiolate was added, and 2.0-ml samples were stored in wax-sealed screw-cap vials at 4 °C.

The labeled antibody for the second part of the IFA reaction was a sheep anti-rabbit globulin which had been conjugated with rhodamine B isothiocyanate (9). Rhodamine B has a fluorescence emission spectrum which falls entirely above 550 nm (13). The labeled globulin will be referred to as SARG-RhITC in subsequent paragraphs. SARG-RhITC was obtained from the Sylvana Co. (Millburn, N.J.) and was stored frozen (−25 °C) in 1.0-ml portions. Just before use, it was thawed and passed through a column (10 by 60 mm) of Sephadex G-25 in the presence of 0.01 or 0.1 M PBS, pH 7.35, to remove unconjugated fluorescing material.

Ion gradients. Antiserum to cell walls was mixed at 1:20 dilution (1:40 was the highest serum dilution producing a + fluorescence reaction) with a series of ion gradients that were prepared by a technique described in a separate communication (10). These gradients consisted of (i) H+ (obtained by varying the proportions of Na2HPO4 and NaH2PO4) giving a pH range of 6.0 to 7.9, (ii) Ce3+ (as CaCl2·2H2O) at 2.5 × 10−4 M to infinite dilution, (iii) Mg2+ (as MgSO4·7H2O) at 5 × 10−4 M to infinite dilution, and (iv) glycine (as a pH mixture of Na2HPO4 and NaH2PO4) at 0.1 M to infinite dilution. In addition to the stated ion, each gradient contained a sucrose gradient, 0.85% NaCl, 1% bovine serum albumin fraction V, and the antiserum.

The gradients were drawn into a long glass tube containing a glass strip coated with dried, heat-fixed bacteria. In addition, pH gradients without sucrose were prepared. Discrete 0.1-ml fractions of the latter were collected, measured with a miniature electrode for exact pH on an expanded scale meter, and dropped on microscope slides containing dried, heat-fixed bacteria (10).

IFA reactions. By using standard microscope slides, the following protocol was followed for testing the effects of pH or buffer molarity on the first part of the IFA reaction (i.e., the coupling of rabbit antibodies to cells). Type E cells were grown for 18 to 24 h at 30 °C in Trypticase-peptone-glucose broth, washed twice in 0.01 M PBS, pH 7.2, and resuspended to 106 cells/ml. About 0.03 ml of cell suspension was air-dried inside a 14-mm etched circle on an acid-dichro-
emat-cleansed glass slide. The dried cells were heat-fixed, dipped three times in distilled-deionized water to remove concentrated salts, and then redried in air. Antiserum fractions from the pH gradient (or preparations of varying buffer molarity) were allowed to react for 30 min at 37 °C in a humid chamber rotated at 100 rpm. Slides were rinsed for 20 min in four changes of 0.01 M PBS, pH 7.25. After being blotted with filter paper, the preparations were exposed to a 1:30 dilution of the tracer (SARG-RhITC) in 0.01 M (or 0.1 M in the case of phosphate variation) sodium phosphate buffer containing 0.85% NaCl and 1.0% bovine serum albumin fraction V (PBS-A) at pH 7.35. Slides were again rotated 30 min in a humid chamber, rinsed, dipped in distilled-deionized water, blotted dry, and mounted in glycerol buffered at pH 7.40.

In determining the effects of pH on the second part of the IFA reaction (i.e., the coupling of the tracer to rabbit antibody), similar procedures were followed, except that the initial reaction was performed with antibody diluted in 0.01 M PBS-A at constant pH 7.25 and the second part of the reaction was performed by using SARG-RhITC at various pH levels. When the effects of varying phosphate molarity were examined, the initial part of the IFA reaction was kept at pH 7.25, and the tracer portion of the reaction was kept at pH 7.35.

Procedures used in examining the effects of continuous linear ion gradients against cells on long glass strips are described elsewhere (10). Briefly, this consisted of incubating a strip of bacterial cells with antibody contained in an ion gradient for 2 h at 37 °C. Further treatment of the strips with SARG-RhITC and rinsing and mounting of cover slips were performed as described for microscope slides.

Measurements of cell fluorescence intensity. A Leitz model SM-M dark-field fluorescence microscope, fitted with an Osram HBO-200 mercury vapor lamp, built-in heat filter, 2-mm Ug-1 transmission
filter, and K-430 suppression filter, was used during all fluorescence excitation. Fluorescence of cells on glass microscope slides was measured with a multipli-
er photometer (Photovolt Corp., model 520 M). The photometer was attached to the microscope by means of a Leitz photometer attachment. Intensity readings for 10 microscopic fields (at 1,000× magnification) on each slide were averaged to obtain a mean reading for the slide. Measurement was accomplished in the following manner. A field of five to eight cells (using the third from largest stop on the micro-attachment) was found by stage manipulation and observation through the split-image focusing telescope, a photometer reading was made, another reading was made on an adjacent field containing no cells and of as near identical background to the first reading as possible, and, finally, a third reading was made on the original field of five to eight cells. The value of the background reading was subtracted from the mean value for the two cell readings to give a fluorescence intensity value for that group of cells. This sandwiching of background reading between two readings of the same field was used to average out any intensity loss during the switch between cell and background readings. All measurements for any single experiment were made within a 4-h period on the same day. Since there was no drift of photometer readings within this period and correlations within experiments but not between experiments were desired, a standard fluorescence source was not used.

Determination of changes in fluorescence intensity of cells on glass strips consisted of traversing along the length of the strip with a dark-field fluorescence microscope using filters and fluorescence excitation as described for glass slides. Visual estimates of changes in fluorescence intensity were recorded on a scale of 4+ to negative, depending on the intensity of the reaction. A 4+ reaction was recorded when cells showed an intense ring of bright yellow-orange fluorescence. A 3+ reaction was indicated by a slightly less intense ring of fluorescence of uneven thickness. A 2+ fluorescence was recorded for cells having a thin ring of moderately bright fluorescence and considerably darkened center. A 1+ reaction was indicated by cells that showed weak color and hazy, indistinct rings. Negative reactions were recorded when cells appeared the same as negative serum controls (strips of cells treated with normal rabbit serum). In some cases the fluorescence reaction did not fall clearly in any one of these categories, but instead exhibited a degree of fluorescence that fell between categories. If, for example, the reaction was between 3+ and 4+ in intensity, it was recorded as 3.5+. This type of notation was also used to show the mean reaction of repeat experiments when the reactions varied slightly.

RESULTS

Influence of pH on the coupling of rabbit antibody to cell surfaces. C. botulinum type E (Kalamazoo) cells were treated with rabbit anti-cell wall serum over a range of H+ concentrations in 0.01 M sodium phosphate buffer.

The IFA reaction was completed by using SARG-RhITC to mark the attachment of the antibody. Direct photometric measurements revealed an interesting kinetic pattern (Fig. 1). At the extremes of the pH range, the fluorescence intensity was at approximately the same level as that of a control treated with normal rabbit serum (NRS) at pH 7.25. This indicates that above pH 7.8 and below pH 6.3 there was little, if any, antibody coupling to the cell surfaces. The optimal pH for the reaction was 7.25. The NRS control showed up as a photometer reading because the cells showed a low-intensity, light tan color under ultraviolet excitation. The photometer did not distinguish this color from the yellow-orange color of specific antibody coupling.

Fig. 2 shows the results when a pH gradient containing anti-cell wall antibody was allowed to react with cells on a glass strip. After

![Fig. 1. Influence of pH on the coupling of rabbit antibody to C. botulinum type E cells. Circles represent the mean intensity of 10 microscopic fields. Squares mark points where the experiment was duplicated and represent the mean of 20 microscopic fields. The oval symbol shows the mean intensity when NRS was substituted for immune serum at the optimal pH of the reaction.](http://aem.asm.org/)

![Fig. 2. Confirmation of the influence of pH on the coupling of rabbit antibody to C. botulinum type E cells by the use of a continuous pH gradient. The histogram shows mean fluorescence intensity along the length of two gradient strips. A strip treated with NRS was uniformly negative. A portion of the photometric curve from Fig. 4 is included for comparison.](http://aem.asm.org/)
treatment with SARG-RhITC to allow visualization of the anti-cell wall antibody, estimates of cell fluorescence intensity were made along the strip. A histogram was constructed from the recorded data which was similar in shape to the curve found by photometric measurement of discrete fractions. The critical portion of the curve found by direct measurement is included in the figure for comparison. The central points of optimal antigen-antibody coupling shown by the two methods varied by 0.05 pH units. The ion gradient method, although less accurate, supported values shown by direct photometric measurement.

**Influence of pH on the coupling of SARG-RhITC to rabbit anti-cell wall globulin.** The influence of pH changes on the second part of the IFA test was studied (i.e., the coupling of SARG-RhITC to rabbit antibodies already attached to cell walls under the optimal condition of pH 7.25). By using the gradient technique, a histogram of estimated fluorescence intensity was obtained with a peak at pH 7.39 (Fig. 3). Although the shape of the histogram resembled that found for the first part of the reaction (see Fig. 2), it was shifted slightly toward a more basic side. Six individual slides were prepared, treated with anti-cell wall antibody, and then treated with SARG-RhITC at pH values in the critical region shown by the gradient technique. Direct photometric measurement (upper curve in Fig. 3) showed a very close correlation to the results of the gradient technique. Optimal pH for this reaction by direct photometric measurement is 7.37. Again, each method supports the other.

**Measurements on the coupling of antibody to cells under varying phosphate concentration.** A gradient of sodium phosphate (0.1 M to infinite dilution) in the presence of rabbit anti-cell wall antibody at pH 7.25 was applied to cells on a glass strip. The second part of the reaction was completed by using SARG-RhITC at pH 7.35 in 0.01 M PBS. Microscope observation showed cells maintaining a 4+ fluorescence intensity along the entire length of the gradient strip; this indicated that sodium phosphate had no influence on the antibody coupling reactions within the concentrations used. A similar strip on which NRS was substituted for antibody showed no specific fluorescence throughout. To confirm this result, photometric measurements were made on cells treated with individually prepared solutions of antibody in 0.01 or 0.1 M sodium phosphate. The effects of combination of these molarities were tested on both the first and second portions of the IFA reaction (Fig. 4). The first two bars were nearly equivalent to extremes of phosphate molarity that were set up on the gradient strip. Although the slides treated with 0.1 M phosphate during the first portion of the IFA reaction had slightly greater fluorescence intensity, the 95% confidence limits shown for the first two bars overlapped. Similarly, the slides treated with 0.1 M phosphate during both portions of the IFA reaction (the third bar) exhibited the highest fluorescence intensity; however, analysis of variance (by randomized complete block design with two factors) indicated no significant differences.
among the four treatment variables. The analysis did show a significant difference between experiments \((P < .005)\). The latter may have been due to differences between bacterial cultures (the experiments were run several months apart) or differences between avidity of RhITC-tagged anti-globulins (several different lots were used). This did not affect the results of treatments, since all tested variables were run on the same day and under identical conditions.

Thus, although there was a slight trend in favor of using 0.1 M phosphate, there were no significant differences in antibody coupling resulting from any of the molar combinations. The ion gradient technique, which initially established the lack of influence of phosphate on the reaction, was again confirmed by photometric measurements.

**Measurements on the coupling of rabbit antibody to cell surfaces in the presence of Ca\(^{2+}\) and Mg\(^{2+}\) gradients.** Gradients of CaCl\(_2\) (2.5 \times 10^{-3} \text{M} to infinite dilution) and MgCl\(_2\) (5 \times 10^{-3} \text{M} to infinite dilution) in the presence of rabbit anti-cell wall antibody at pH 7.25 were applied to cells on a glass strip. The strips were examined by dark-field fluorescence microscopy after completing the second (SARG-RhITC) portion of the IFA reaction at pH 7.35. Uniform 4+ fluorescence intensity along the entire length of each strip indicated that Ca\(^{2+}\) and Mg\(^{2+}\) have no influence on the coupling of antibody to the cell walls. NRS control strips were negative.

**DISCUSSION**

Experiments in this paper show the effect of pH on the coupling of antibody with antigen. The H\(^+\) effect should not be confused with the higher pH values needed to attach tracer fluorochrome to antibody globulin nor with any influence of pH on emission intensity of the fluorochrome after the antigen-antibody reaction has occurred. In the former case, the fluorochrome was attached commercially before receipt of the RhITC-labeled sheep anti-rabbit globulin. As for the latter, any possible pH effect on fluorochrome emission intensity was kept constant by always mounting cover slips over the completed reaction by using glycerol buffered at pH 7.40.

In the past, little or no consideration has been given to possible differences in pH optima between the two parts of the IFA reaction. Until now no measured data on pH effects during FA reactions have been reported in the literature. The instrumental data in this paper show that pH has a strong influence on the antigen-antibody reactions of both portions of the IFA test. The effect is confirmed by the use of a slightly less accurate but more rapid ion gradient technique for studying reaction kinetics. The optimal pH for coupling of rabbit antibody to C. botulinum cells was 7.25, whereas the coupling of tagged sheep anti-rabbit globulin to rabbit antibody (the second portion of the reaction) occurred optimally at pH 7.37. These optima fall within relatively narrow limits. Running a reaction at pH values as little as 0.1 to 0.15 units off from the optima will significantly reduce the intensity of the reaction, particularly if there has been deviation from both optima within the same IFA test.

Slight shifts from optimum may not seriously affect FA tests for microorganisms in which fluorescein isothiocyanate (FITC)-tagged globulin is used directly on cultured material. However, when fluorescent labels of lesser intensity are used (as in the present case), pH optima may be very important. Also, in instances where tracer work is being attempted in tissues, fluorescence is generally reduced; therefore, pH optima may influence the outcome even though the stronger FITC label is used. In the present case, the RhITC label was selected because it contrasted with both autofluorescence of the bacterial cells and intense, green autofluorescence of fish tissues, which frequently harbor the type E organism.

The reason for the difference in pH optima between the two portions of the IFA test is not known; however, either (or both) of two mechanisms could be operative. It is known that the isoelectric point of bovine albumin conjugated with fluorescein differs by 0.13 pH unit from that of unconjugated bovine albumin (11). Since the rabbit anti-cell wall antibody used in this study was unconjugated, whereas the sheep anti-rabbit globulin was conjugated with a rhodamine dye, the pH shift may have been due to dye conjugation. On the other hand, the animal species in which the anti-globulin is produced may influence the pH optima of antigen-antibody reactions for that globulin. Some support for this theory is found in the fact that the reported pH of rabbit blood differs from that of sheep blood. Tabulated values collected from a variety of sources indicate that the mean pH for rabbit blood is 7.35 (with a range of 7.21 to 7.57); the mean for sheep blood is 7.44 (with a range of 7.32 to 7.57) (15).

Data in this paper show that there is little coupling of antibodies with cell wall antigens when the pH is below 6.3 or above 7.8. In one respect (i.e., a falloff in antigen-antibody coupling above and below a particular pH range),
Lysozyme-rabbit albumin complexes dissociate in a stable state that is reported for precipitin complexes. Between these reactions, in general, a uniformly high quantity of precipitate throughout their precipitation pH range, whereas this IFA reaction had a relatively narrow pH optimum, with the quantity of antigen-antibody coupling falling away on either side of the peak. The shape of the curve in this IFA reaction probably results from a multitude of antigens and antibodies coupling in concert, whereas precipitin curves in the cited studies result from essentially pure, single antigen-antibody interactions. Multiple antibodies are present because the cell wall of the immunizing organism contains a number of antigenic determinants. These antibodies have been demonstrated by cross-reaction and absorption with closely related bacteria (Robohm, manuscript in preparation).

Although a phosphate buffer of 0.01 M is generally used for fluorescence conjugates, the IFA reaction in this instance was not affected by ranges of sodium phosphate from 0.01 to 0.1 M. In addition to a slight (but statistically nonsignificant) trend of measured values favoring the use of 0.1 M buffer, it is easier to use the 0.1 M buffer for FA work. Reactants prepared in 0.01 M buffer and stored overnight at 4 C frequently shift in pH by the next morning. The use of 0.1 M buffer seems to allow better pH stabilization for globulins used in the current work.

The effects of Ca\(^{2+}\) and Mg\(^{2+}\) gradients on the anti-cell wall reactions were measured because of the effects these ions are known to have on the antigen-antibody complement system for the lysis of red blood cells. As little as 1.5 \(\times 10^{-4}\) M CaCl\(_2\) and 5 \(\times 10^{-4}\) M MgSO\(_4\) have been shown to be necessary for the complement system (reviewed by Mayer, 7). These ions apparently have no effect on the IFA reaction for \(C.\) botulinum\; type\; E in the range of 5 \(\times 10^{-3}\) M to infinite dilution.

**ACKNOWLEDGMENTS**

This work was performed at the former Technology Laboratory, National Marine Fisheries Service, Ann Arbor, Mich. I thank Arthur G. Johnson, Dept. of Microbiology, The University of Michigan, for critical review of the work, and John T. Graikoski, National Marine Fisheries Service, Milford, Conn., for the \(C.\) botulinum\; type\; E (Kalamazoo) culture.

**LITERATURE CITED**

1. Cherry, W. B., M. Goldman, T. R. Carksi, and M. D. Moody, 1960. Fluorescent antibody techniques. Public Health Service Publ. no. 729. U.S. Government Printing Office, Washington, D.C.
2. Coons, A. H. 1958. Fluorescent antibody methods, p. 399-422. In J. F. Danielli (ed.), General cytochemical methods, vol. 1. Academic Press Inc., New York.
3. Goldman, M. 1968. Fluorescent antibody techniques. Academic Press Inc., New York.
4. Kawamura, A. Jr., ed. 1969. Fluorescent antibody techniques and their applications. University Park Press, Baltimore.
5. Kleinschmidt, W. J., and P. D. Boyer. 1952. Interactions of protein antigens and antibodies. I. Inhibition studies on the egg albumin-anti-egg albumin system. J. Immunol. 62:247-255.
6. Marrack, J. R. 1958. The relation of the rates of flocculation and amounts of precipitate in precipitin reactions to the concentration of hydrogen ion and neutral salts. Immunology 1:251-267.
7. Mayer, M. M. 1958. Studies on the mechanism of hemolysis by antibody and complement. Progr. Allergy 5:215-270.
8. Nairn, R. C. 1969. Immunological tracing: general considerations, p. 129 and 303. In R. C. Nairn (ed.), Fluorescent protein tracing, 3rd ed. The Williams and Wilkins Company, Baltimore.
9. Riggs, J. L., R. J. Seiwald, J. H. Burckhalter, C. M. Downs, and T. G. Metcalf. 1958. Isothiocyanate compounds as fluorescent labeling agents for immune serum. Amer. J. Pathol. 34:1081-1097.
10. Robohm, R. A. 1973. Gradient technique to test the effects of substances on fluorescent antibody reactions. Appl. Microbiol. 27:259-261.
11. Schiller, A. A., R. W. Schayer, and E. L. Hess. 1953. Fluorescein conjugated bovine albumin—physical and biological properties. J. Gen. Physiol. 36:489-506.
12. Schmidt, C. F., W. K. Nank, and R. V. Lechowich. 1962. Radiation sterilization of food. II. Some aspects of growth, sporulation and radiation resistance of spores of Clostridium botulinum\; type\; E. J. Food Sci. 27:77-84.
13. Silverstein, A. M. 1957. Contrasting fluorescent labels for two antibodies. J. Histochem. Cytochem. 5:94-95.
14. Singer, S. J., and D. H. Campbell. 1955. Physical-chemical studies of soluble antigen-antibody complexes. IV. The effect of pH on the reaction between bovine serum albumin and its rabbit antibodies. J. Amer. Chem. Soc. 77:3504-3510.
15. Spector, W. S., ed. 1956. Handbook of biological data. W. R. Saunders Co., Philadelphia.