Fluorometric estimation of antigens (antibodies)*

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A method for the titration of antigens based on separation by filtration through millipore filters of the complex of antigen with fluorescent isothiocyanate-labeled antibodies from free labeled antibodies and nonspecific γ-globulins is described. The fluorescence of the antigen-antibody complex is measured directly on the millipore filters using the Turner fluorometer equipped with a paper chromatogram door. The limit of sensitivity of the method is about 0.05 μg of antigen protein.

Quenching of fluorescence by nonlabeled antibodies can be used for the titration of antisera. An example of the application of the method for titration of rabies virus antigen is also presented.

The fluorescent antibody technique has been widely used for the localization of antigens (antibodies) in tissues and cells. A fluorescent cell-counting technique for the assay of infective virus particles has been developed 1-4. The immunofluorescent method combined with microphotomomery has been used for the differentiation of closely related viruses 5. Recently a cellulose acetate fluorescent spot test for titration of antigens (antibodies) has been developed 6. The immunofluorescent method combined with microphotomometry has been used for the differentiation of closely related viruses 5. In addition, fluorescent isothiocyanate (FITC) labeled antibodies represent a potential tool for a direct fluorometric method for estimation of antigens. The prerequisite for the development of such a method is to find a convenient way to separate the antigen—conjugated antibody complex from free antibodies and nonspecific conjugated γ-globulins, and to obtain the antigen-antibody complex in a small volume. Filtration through millipore filters was expected to serve the purpose. Furthermore, it should be possible to titrate antibodies (not conjugated) on the basis of competition between unlabeled and labeled antibodies for the same antigenic sites, resulting in a decrease of fluorescence of the antigen-antibody complex.

Materials and methods

Various dilutions of the antigen solution (see text to the figures) were mixed with a constant amount of FITC-conjugated antibody solution and with 0.5 ml. of normal activated (56 °C, 30 min.) calf serum. In experiments in which serum proteins were used as antigen, the addition of calf serum was omitted. The calf serum has been used to supress a possible binding of conjugated globulins to cellular debris possibly present in virus suspensions. The mixtures, the total volume of which should not exceed 3 ml. (= maximal capacity of the filtering device), were incubated at 37°C for 30 min. and then kept at 4 °C overnight. In experiments in which antibodies were to be titrated, constant amounts of both antigen and FITC-conjugated antibodies were mixed with various dilutions of the antiserum tested. The solutions of antigen, labeled antibody, antiserum and calf serum, respectively, were clarified by centrifugation or by filtration through millipore filters (50–220 μm) before beginning the test. Appropriate non-infected control material samples were included in tests for viral antigen. On the second day, the individual suspensions were filtered through millipore filters of 220 μm pore size (with smaller pore size filters it was difficult to prevent clogging) previously washed with 1 ml. of normal calf serum (2% solution of bovine hemoglobin in the case of bovine globulin as antigen) to saturate the protein binding sites of the filters 7. The I.E.C. filtering centrifuge tubes (Millipore, Catalogue No. XX 62 013 00) were used for filtration (centrifugation at 2,500 r.p.m. for 15 min.). The filtrates were discarded and the filters were washed twice with buffered (pH 7.2) isotonic saline and centrifuged at 2,500 r.p.m. for 7 minutes. The washed millipore filters were then removed from the filtering device and stored for further evaluation.

The fluorescence of the microprecipitate was measured directly on the millipore filters using the Turner fluorometer type 110, equipped with a paper chromatogram door. Narrow band-pass filters, chosen

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5 C. Kenz, Virology 24, 672 [1964].
according to the excitation and emission spectra of FITC-labeled globulins, with maximal transmittance at 430 and 520 m\(\mu\) were used as primary (excitation) and secondary (emission) filters, respectively. The range of sensitivity of the measurements was adjusted by Bausch & Lomb gray filters of known transmittance used as additional primary filters.

**Results and discussion**

A precipitin curve obtained by the method presented, showing a decrease of fluorescence in the antigen excess region, is presented in Fig. 1. Fig. 2 demonstrates the high sensitivity of the method which covers the lower limit of the sensitivity described for the complement fixation test. The best results (linearity of the calibration curve) were obtained in a region of low antigen concentration at which no visible precipitation occurred. Results with rabies virus antigen (tissue culture fluid from hamster kidney fibroblasts infected with the PM strain of rabies virus with a titer of \(10^{4.5}/0.03\) ml.

![Fig. 1. Precipitin curve. Antigen: Globulin fraction obtained from bovine serum by precipitation with \((NH_4)_2SO_4\) (satisfaction to 50%); 1 ml of various concentrations was used. Conjugated antibody: Globulin fraction of rabbit antiserum against bovine serum globulin (0.1 ml.; Microbiological Associates Inc., Bethesda, Md.).](image)

![Fig. 2. Calibration curves for two antigens: Globulin fraction from bovine serum (—•—, bottom abscissa) (= ascending part of the curve from Fig. 1), and rabies virus antigen (— ■ —, top abscissa) (0.1 ml. volumes of conjugated anti-rabies globulin, Baltimore Biological Lab., were used). Blanks for rabies virus consisted of the corresponding volume of normal hamster kidney tissue culture fluid treated in the same way as the virus containing fluid.)](image)

![Fig. 3. Titration of antisera against bovine globulins (—•—, 0.5 ml. of serial twofold dilutions of the antiserum from Baltimore Biol., Lab., constant amounts of antigen (6 m\(\mu\)) and conjugated globulin (0.1 ml.) were used) and against rabies virus antigen (— ■ —, 1 ml. of serial twofold dilutions of a monkey antiserum, 2 ml. of the antigen suspension and 0.1 ml. of the conjugated globulin solution were used).](image)

![It will be shown elsewhere that the majority of material giving a positive reaction in this test is due to a specific “soluble” antigen(s) of rabies virus.](image)

observed in control experiments using FITC-conjugated globulins from normal equine, rabbit and monkey sera (used in the same or even higher concentrations in comparison with the corresponding specific labeled globulin preparations) with both antigens tested.

An example of titration of antisera is given in Fig. 3. It is evident that the titers of various antisera can be easily compared by finding the dilutions at which a 50% reduction of fluorescence occurs.

It may be concluded that the use of FITC-conjugated (or isotopically labeled) antibodies in combination with filtration through millipore filters offers a sensitive and precise direct method for quantitative immunochemical studies and represents a convenient tool for titration of antigens and antibodies. This has been found useful for titration of rabies viral antigen and might also be useful for titration of other virus antigens.

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