QUANTIFICATION OF THE ANTIGEN-ANTIBODY REACTION BY THE POLARIZATION OF FLUORESCENCE*

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The basic theory concerning the polarization of fluorescence was developed in a series of important papers by Perrin (1926). Perrin's results have been used experimentally by Singleterry and co-workers (1951) and greatly extended, both theoretically and experimentally, by Weber (1952). Subsequent applications by Laurence (1952) and Steiner (1957) follow implicitly from the work of Weber.

The concept underlying previous work by Dandliker and Feigen (1961) and the present results is to utilize the change in rotary diffusion constant which occurs when an antigen and antibody combine in solution. An essential feature of the method is that either the antigen or antibody is made fluorescent depending upon which component is to be detected. This feature makes it possible to follow the reaction in spectral regions where adventitious fluorescence is of minor importance and also permits a choice of fluorescence lifetime, appropriate to the range of molecular size involved; it is thus distinct from other fluorescence techniques in use, cf. Coons, et al. (1941), Boroff and Fitzgerald (1958) and Velick, et al. (1960).

EXPERIMENTAL. Crystalline ovalbumin was labeled with fluorescein using fluorescein isothiocyanate (Riggs, et al. 1958); the product contained between

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one and two fluorescein molecules per molecule of protein assuming the molar extinction coefficient of free and bound fluorescein to be the same. Eight albino rabbits, weighing about six pounds each, were immunized to fluorescein-labeled ovalbumin (F-ovalbumin) by a series of twenty intravenous injections of 10 mg. each administered on alternate days. One week after the last injection, blood was drawn by cardiac puncture and, after clotting, the serum was separated by centrifugation. A γ-globulin fraction from pooled serum was prepared by two successive precipitations in one-third saturated ammonium sulfate. For control purposes, γ-globulin was also prepared from normal animals. The immune globulin preparation contained 19% specifically precipitable anti-F-ovalbumin as estimated at optimal proportions by means of the quantitative precipitin method; this antibody also reacted strongly with native ovalbumin.

Measured volumes of antigen solution were added from a microburette to a constant quantity of antibody contained in a cuvette. The intensity and polarization of fluorescence were measured in a modified Brice-Phoenix apparatus using the unpolarized 4358 Å mercury line for excitation.

RESULTS AND DISCUSSION. The reaction between F-ovalbumin and its antibody produces two fluorescence effects. First, there is a pronounced diminution of the fluorescence due, no doubt, to the close juxtaposition with many atomic groupings of the antibody, thus establishing favorable conditions for loss of the electronic excitation energy before fluorescence takes place; possibly a transition to the triplet state is involved. The second effect is the change in polarization of fluorescence caused by the increase in relaxation time.

For quantitative puposes, it is convenient to define the polarization (p) and a parameter (Q) which is proportional to fluorescence intensity divided by incident intensity. If the vertical and horizontal components in

the fluorescent light are denoted by V and H respectively, and if M_F denotes the molar concentration of fluorescent antigen, then $p = \frac{V - H}{V + H}$ and $Q = \frac{V + H}{M_F}$.

In a typical experiment (Figure 1), 4 ml. of antibody solution was mixed with portions of F-ovalbumin to give final concentrations ranging upward from about 5×10^{-9}M.

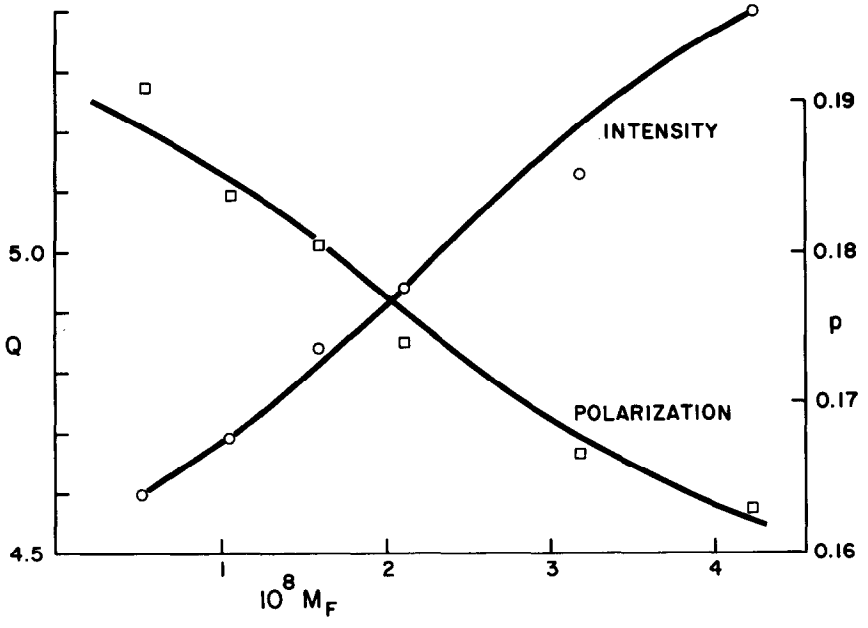

Fig. 1. Q and p as a function of M_F (see text) for a solution containing 7 μg. of precipitable anti-F-ovalbumin / ml. The curves were calculated from the constants derived from Figure 2.

If it is assumed that the bound form of the antigen may be characterized by a limiting value for Q and p equal to k_b and p_b respectively, and if the free form of the antigen is similarly characterized by k_f and p_f, then a simple mass law analysis of the data may be made according to the reaction $F + Ab \rightleftharpoons FAb$. The association constant, $K = \frac{(FAb)}{(F)(Ab)}$

For this analysis, all concentrations are measured in terms of the molarity of antigen. With these assumptions, it is possible to express results

(Figure 2) as suggested by Scatchard (1949). The data for both intensity and polarization are concordant and indicate some spread in the association constants. In principle, the curvature of the Scatchard plot should be capable of yielding information concerning the range of association constants, but the analysis requires very accurate data.

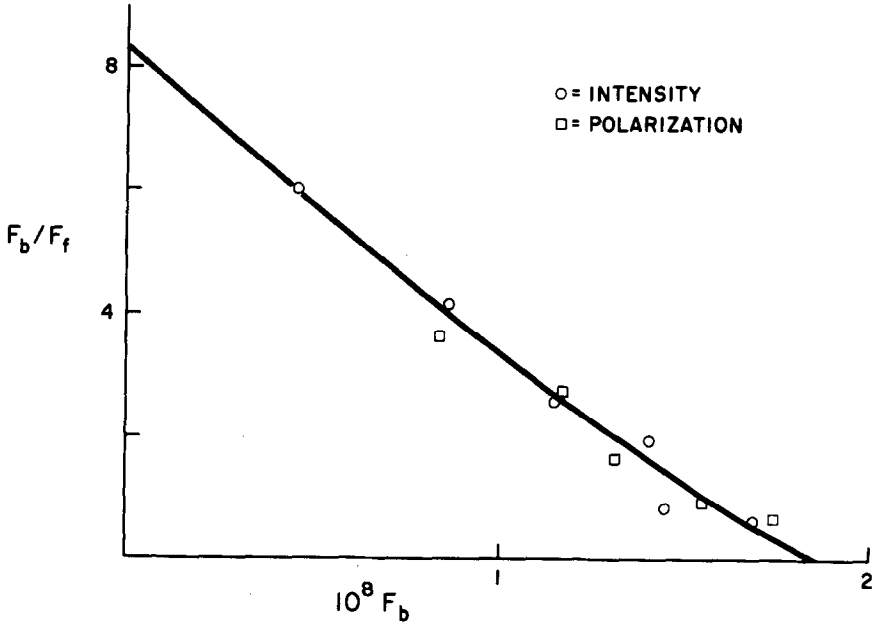

Fig. 2. Scatchard plot of the data from Fig. 1. F_b and F_f are the molar concentrations of F-ovalbumin, bound and free respectively. The association constant, $K = 4.3 \times 10^8$ and the maximum value of F_b is 1.9×10^{-8}.

For the method described here to have general applicability, the fluorescent antigen or antibody must be capable of reacting in solution with antibody to the native antigen or with the native antigen itself, respectively. Figure 3 gives the quantitative results for the system F-ovalbumin and antibody to native ovalbumin (anti-ovalbumin). There are distinct and important differences between the behavior of this system and that shown in Figure 1. First of all, there is little or no variation of Q, i.e. no quenching

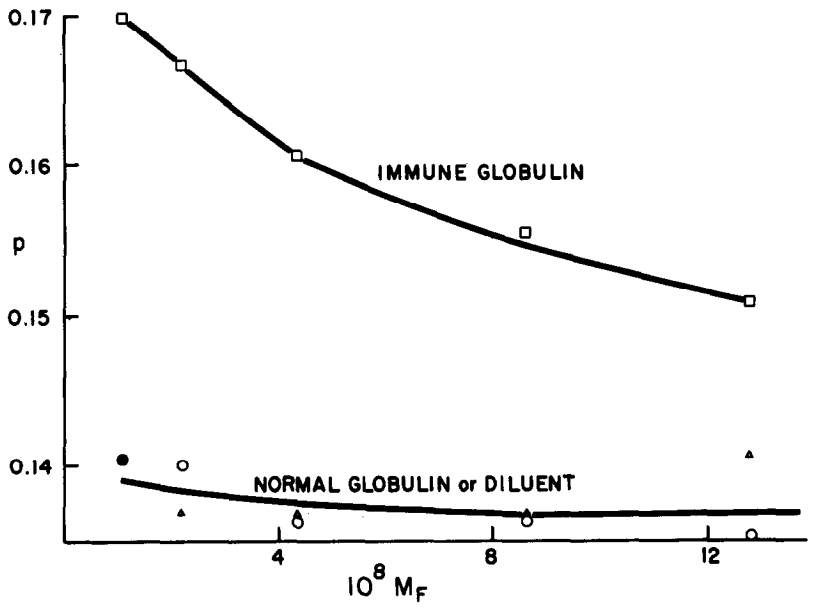

Fig. 3. Polarization as a function of M_F. F-ovalbumin was added to anti-ovalbumin (squares) or to normal γ-globulin (circles) of the same protein concentration or to diluent, i.e. 0.15M sodium chloride, 0.01M disodium hydrogen phosphate and 0.005M sodium dihydrogen phosphate (triangles).

or enhancement of fluorescence. Physically, this behavior has a simple and straightforward interpretation because the antibody in this case has no site for the specific binding of fluorescein. The polarization effects are similar to those in Figure 1. No detectable interaction at these concentrations between normal globulin and antigen was found, the results being the same within experimental error as those obtained by addition to buffered saline.

SUMMARY. We have concluded that it is possible to determine by measurements of fluorescence polarization two important parameters, namely the equilibrium constant and the combining capacity characterizing the antigen-antibody reaction. The combining capacity is proportional to the number of antibody sites in a preparation and the equilibrium constant, together perhaps with certain kinetic quantities, constitutes a quantitative measure of

avidity. Further results will show to what extent the presence of the fluorescent label interferes in the reaction, but, in any case, it appears that the effect is relatively small.

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