ANTI-FLUORESCEIN ANTIBODY OF HIGH AFFINITY AND RESTRICTED HETEROGENEITY AS CHARACTERIZED BY FLUORESCENCE POLARIZATION AND QUENCHING EQUILIBRIUM TECHNIQUES*

A. J. Portmann, S. A. Levison† and W. B. Dandliker

Department of Biochemistry
Scripps Clinic and Research Foundation
La Jolla, California 92037

Received February 24, 1971

Summary: An unusually high affinity constant ($K = 6 \times 10^{10} M^{-1}$) together with a lack of heterogeneity ($a = 1.0$) has been found in an antifluorescein antibody, using extremely sensitive instrumentation in connection with fluorescence polarization and quenching techniques. No systematic variation of the K and a values could be detected over a range of antibody concentrations differing more than 60-fold. A simple method for estimating titers of high-affinity antibodies from polarization titration curves is also described.

Introduction: Extensive studies on antibodies directed against haptenic determinants have been reported in which most of these antihaptens could be characterized by an average association constant, K_o, of the order of 10^6 to 10^8 M^{-1}. Occasionally slightly higher values were obtained, but it seems that the sensitivity of the measurements made has put an upper limit to the accurate determination of association constants at about 10^9 M^{-1} (1-4). In one instance where an approximate K of $\sim 10^{11}$ has been published (5), no data were given which would indicate the degree of accuracy of this value. Recently, a K_o of this order also has been found utilizing a phage inhibition technique (6).

We now report the accurate determination of an association constant of 6×10^{10} M^{-1} by optical techniques for the reaction between an antifluorescein antibody and fluorescein as a hapten. In contrast to other studies, this K value has been shown to hold over a range of antibody concentrations varied more than 60-fold. Both methods used, i.e. fluorescence polarization and fluorescence intensity measurements, yielded essentially linear Scatchard plots together with a heterogeneity constant close to 1.0

* Supported by the National Science Foundation, (NSF GB15594).
† Awardee of National Institutes of Health special fellowship (NIH 1FO3-AM-42, 568-01).

as determined from Sips plots, implying that this particular antibody preparation is of restricted heterogeneity as contrasted to ordinarily obtained antibody.

Materials: Antibody vs. fluorescein was obtained from rabbits immunized with fluorescein covalently bound to an ovalbumin carrier, as described in a companion paper (7). Fluorescein was purified by ion exchange chromatography as described previously (8).

Tris buffered saline (0.15 M NaCl, 0.01 M tris, pH 7.2) was used throughout this study as a solvent.

Methods: Titrations were carried out in a square 1 cm cuvette containing 3 ml antibody solution. Fluorescein was added in microliter amounts; more than 60 µl were never added in order to avoid dilution effects.

Polarization (Fig. 1) and intensity (Fig. 2) of the fluorescence were determined in a direct read-out polarometer‡)(9) of very high sensitivity where a fluorescein concentration of 6×10^{-11} M still gave useful readings.

Association and heterogeneity constants were derived from the data as outlined by Dandliker et al. (10), using the following relationships:

$$\frac{F_b}{F_f} = \frac{Q_f}{Q_b}\left(\frac{p - p_f}{p_b - p}\right) = \frac{Q_f - Q}{Q - Q_b} \qquad (1)$$

where F = fluorescein concentration, p = polarization, Q = molar fluorescence, i.e. intensity divided by fluorescein concentration, and the subscripts $_f$ and $_b$ refer to the free and bound state, respectively*), while Q and p (without subscripts) are the values measured at any given point of the titration curve.

Equation (1) relates the data from either polarization or intensity measurements to the ratio of bound to free hapten. The association constant, K, as well as the maximum value of F_b, namely $F_{b\,max}$ (which is equivalent to the concentration of available binding sites), can then be obtained through the Scatchard equation (Fig. 3):

$$F_b/F_f = K(F_{b\,max} - F_b) \qquad (2)$$

The heterogeneity index, a, is determined using a linear form of the Sips equation, as illustrated in Fig. 4:

$$\log F_f = \frac{1}{a}\log\left(\frac{F_b}{F_{b\,max} - F_b}\right) - \log K \qquad (3)$$

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*) Q_f and p_f were measured directly; Q_b and p_b were determined by extrapolation according to Dandliker et al. (10).

‡) For some time we have been using "polarometer" to denote an instrument for measuring the degree of polarization as contrasted to optical rotation.

Theoretical curves, assuming (a) homogeneous antibody population, or (b) two species of antibodies of different affinity and in variable relative amounts, were obtained with the help of a digital computer, programmed in Fortran IV.

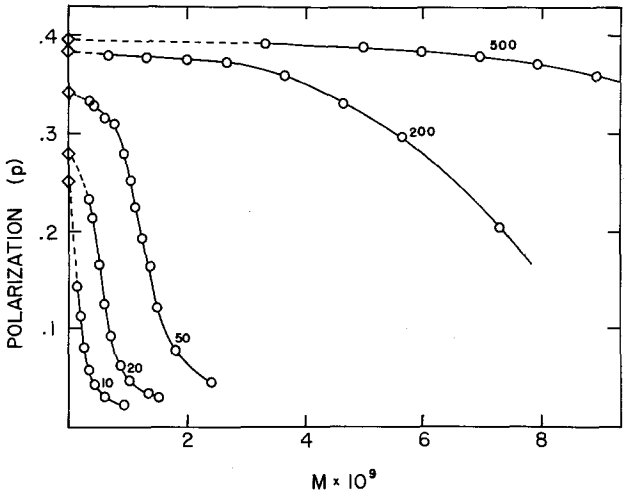

Figure 1. Fluorescence polarization titration curves of various relative amounts (small numbers) of immunospecifically purified antifluorescein antibody performed in tris buffered saline, pH 7.2 at 22°. ○ , experimental p values; ◇ , extrapolated initial p values (10). $p_f = 0.017$; $p_b = 0.400$; $Q_f/Q_b = 15$.

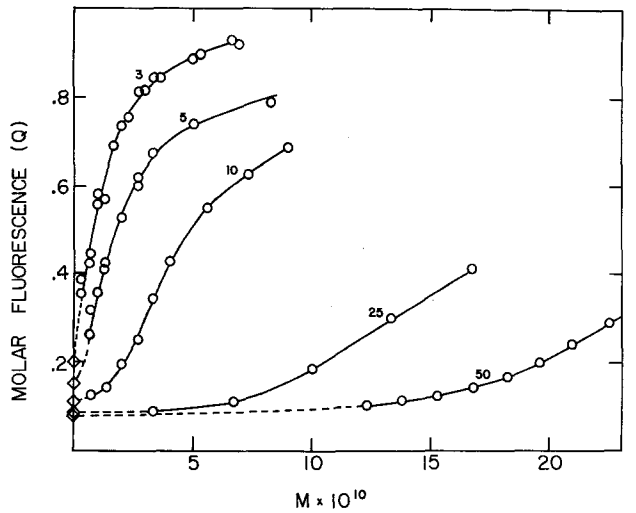

Figure 2. Molar fluorescence intensity titration curves of various relative amounts (small numbers) of immunospecifically purified antifluorescein antibody, performed in tris buffered saline, pH 7.2 at 22°. M, molarity of fluorescein added; ○, experimental intensity values divided by M (arbitrary units, chosen so that $Q_f = 1.0$); ◇ , extrapolated initial Q values (10). $Q_b = 0.067$.

Results: Antibody solutions ranging in relative concentrations from 3 to 200 were titrated following both fluorescence polarization (Fig. 1) and intensity (Fig. 2). The evaluation of data sets from 14 titrations yielded essentially linear Scatchard plots (Fig. 3) over the entire range of concentrations studied. Values for $F_{b\ max}$, i. e. concentrations of available binding sites, were found to range from 10^{-10} to 7×10^{-9} M.

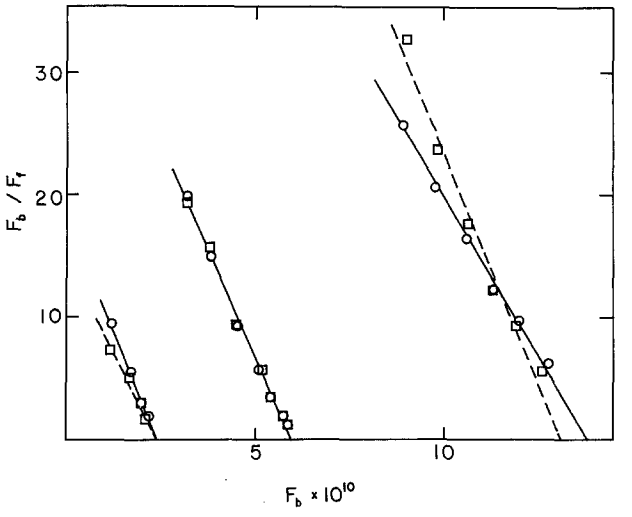

Figure 3. Scatchard plots from titration data of 3 different antibody concentrations; O , using Q values; □ , using p values. Slope of the straight lines = -K; intercept with horizontal axis = $F_{b\ max}$, i. e. titer of antibody.

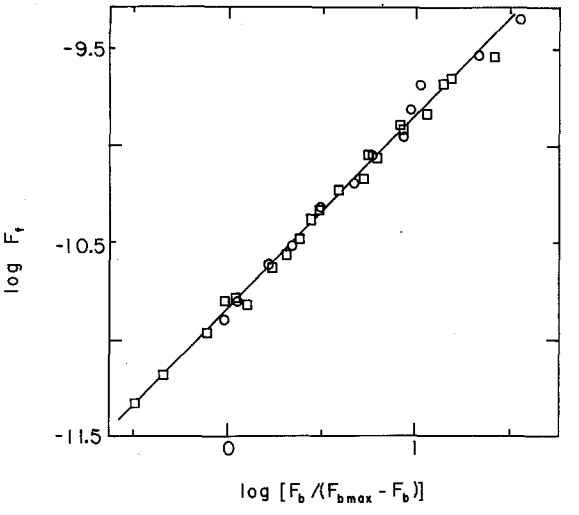

Figure 4. Sips plot from titration data of 3 different antibody concentrations; O , using Q values; □ , using p values. Slope of the best fitting straight line (least square method) = 1.0, implying absence of heterogeneity. At midpoint of titration (horizontal axis = 0): log F_f = -log K.

The average value (± standard deviation) for the association constant was determined as 6.1 ± 1.7 M^{-1} from intensity data, and 6.5 ± 1.6 M^{-1} from polarization data, indicating that there is no significant disagreement between the two methods used.

When the data was plotted according to the Sips equation (Fig. 4), confirmation of these results has been obtained, individual values for K falling into the same range as above and those for the heterogeneity constant being close to unity, resulting in an average a = 1.0.

Discussion: Although $F_{b\ max}$, i.e. concentration of binding sites or, in other words, the titer of the antibody was determined throughout this work from Scatchard plots, it became evident that it is possible to estimate the titer fairly accurately by even a superficial examination of some of the titration curves obtained by the fluorescence polarization method. In those cases where the polarization curves display a sharp break, i.e., at the higher antibody concentrations, the fluorescein concentration at the breaking point can be set equal to $F_{b\ max}$. Justification of this procedure also has been obtained from computer generated data: it has been shown that the necessary shape of the titration curve will be realized at antibody concentrations about 100 fold (or more) larger than the reciprocal of the association constant. In the case of antibody of high affinity there is no difficulty in meeting this requirement, the minimum concentration of the antibody reported here being only about 10μ g/ml of IgG. Titers so determined will be subject to an error of less than 5%.

The fact that a heterogeneity constant of 1.0 was found does not necessarily mean that this antibody preparation is completely homogeneous. Computer generated data have shown that the presence of another antibody would remain undetected if this second antibody were to have an affinity constant about 1000 times or more smaller than the high affinity antibody. The titration of the strong antibody would then be essentially complete before any noticeable amount of the weak antibody is bound, thereby masking the presence of the weak antibody. Since the protein content of our preparation, as determined by optical density measurement, is about twice the amount derived from the titer (assuming bivalent antibody), the experimental results allow the conclusion that about half of our preparation consists of a population of very high affinity antibody which in itself is homogeneous with respect to K, but it cannot be decided at present if the remaining material consists of antibodies with drastically smaller affinity or of antibodies inactivated during the process of immunospecific purification.

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