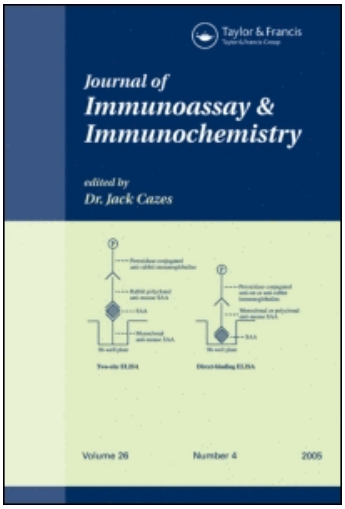

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Determination of Antibody Affinity

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I. INTRODUCTION

As the science of immunology matures, there is increasing need for its experimental observations to be expressed in quantitative terms. For antigen–antibody interactions, this means that the activity and binding strength of antibodies should be described in terms of their equilibrium affinity constant (Steward and Steensgaard, 1983). For instance, when attempts are made to mimic protein epitopes by means of synthetic peptides, the best criterion for assessing how closely the peptide matches the corresponding epitope in the cognate protein is to measure the comparative binding affinities of an antibody capable of reacting both with the intact molecule and with the synthetic fragments. Changes in the equilibrium affinity constant and kinetic rate constants of an antibody are very sensitive indicators of alterations in the structure of either the antigen or the antibody. This is why these constants are useful parameters for the quality control of engineered antibodies and recombinant proteins (Marks et al., 1992). The affinity of antibodies is also a crucial factor that determines how well they will perform in different types of immunoassays. Antibodies of higher affinity will allow smaller amounts of an antigen to be detected (Stewart and Lew, 1985; Yolken, 1985) whereas antibodies of moderate affinity are preferable for use in affinity chromatography, since they will allow the antigen to be dissociated more easily after formation of the complex.

The interaction between antigen and antibody at equilibrium may be expressed as

$$A + B \underset{k_d}{\overset{k_a}{\rightleftarrows}} AB$$

where A represents free antigen, B free antibody, and AB the antigen–antibody complex at equilibrium. The equilibrium association constant may be calculated from:

$$K = k_a/k_d = \frac{[AB]}{[A][B]}$$

Although the equilibrium may also be defined from the dissociation of the complex ($K_{diss} = 1/K = k_d/k_a$), the association equilibrium constant that has the dimension of l/mol seems a more natural measure of antibody affinity, since it increases in magnitude when the affinity is higher

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and a greater proportion of the antibody is bound. An antibody of very high affinity, for instance, would thus be expressed as $K = 10^{10}\ M^{-1}$ or $K_{diss} = 10^{-10}\ M$.

II. DEFINITIONS AND BASIC EQUATIONS

Affinity is a thermodynamic expression of the primary interaction of a single epitope with a single paratope and, in principle, the concept should be used only for a monovalent antigen binding to a single Fab fragment. For multivalent antigens and antibodies, the antibody-binding capacity should be expressed by the term *avidity*, which incorporates the role played by antigen and antibody valency in the observed interaction. The importance of the valency term in affinity measurements has been discussed by Underwood (1988). In the present chapter, the valency terms will be introduced in all equations to emphasize their contribution to the measured parameters.

A variety of symbols have been employed to express the different parameters used in affinity calculations, which is somewhat unfortunate, since it does not facilitate the reading of the literature. When the symbols used by Hardie and Van Regenmortel (1975) are employed, the association equilibrium constant K of an antibody may be expressed in terms of the mass action law as follows:

$$K = \frac{sx}{(As - ny)(Bn - ny)} \tag{1}$$

and

$$K = \frac{ny}{(As - sx)(Bn - sx)} \tag{2}$$

where A = total antigen concentration (moles/liter)
s = antigen valence
As = total antigen sites (mole sites/liter)
B = total antibody concentration (moles/liters)
n = antibody valence
Bn = total antibody sites (mole sites/liter)
x = bound antigen concentration (mole/liter)
y = bound antibody concentration (mole/liter)
$ny = sx$ = total bound sites

Equations (1) and (2) are usually rearranged into forms suitable for the graphic representation of binding data. By dividing the numerator and denominator of Eq. (1) by B, one obtains:

$$K = \frac{x/B}{(A - x)(n - sx/B)} = \frac{r}{c(n - sr)}$$

and

$$\frac{r}{c} = K(n - sr) \tag{3}$$

where $r = x/B = ny/sB$: ratio of bound antigen to total antibody; $c = A - x$: free antigen concentration.

Equation (3) allows the construction of Scatchard plots of the type r/c versus sr from which the value of K (as the slope) can be obtained (Fig. 1a). In such plots, when $r/c \rightarrow 0$, $n - sr \rightarrow 0$ and $sr \rightarrow n$. When the antigen is a monovalent hapten ($s = 1$), the value of the antibody valence can be directly obtained by extrapolation. When antibody preparations purified by affinity

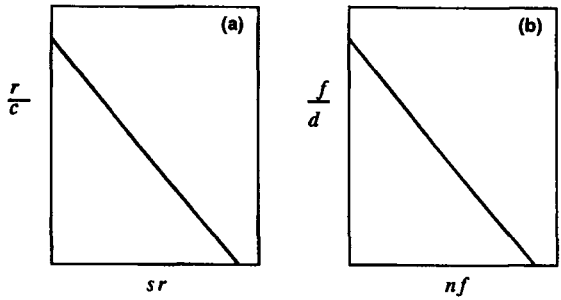

Figure 1 (a) Schematic graphic representation of Eq. (3): r = ratio of bound antigen per total antibody; c = concentration of free antigen (mol/L); s = antigen valence. The extrapolation to the abscissa gives the value of n, whereas the extrapolation to the ordinate gives the value of Kn. (b) Representation of Eq. (4): f = ratio of bound antibody per total antigen; d = concentration of free antibody (mol/L); n = antibody valence. The extrapolation to the abscissa gives the value of s, whereas the extrapolation to the ordinate gives the value of Ks. In both a and b, the affinity constant K is given by the slope.

chromatography are used, it is commonly found that a certain proportion of the antibody used in the binding test has been denatured during the dissociation step and is no longer capable of binding. In the presence of nonfunctional antibody molecules, r/c versus r plots do not extrapolate to $n = 1$ or $n = 2$ but to lower values. In such a situation, it is possible to correct the data by calculating the concentration of functionally active antibody B_{corr} from the relation:

$$B_{corr} = B_{total} - B_{total}(n - n_1)/n$$

where n_1 is the extrapolated value found in the r/c versus r plot, and n is the theoretical value (1 or 2). Once this corrected value of B has been obtained, it can be used for recalculating d, y, f, r, and K.

For large multivalent antigens, it is not possible to measure experimentally the quantity c (or x), since only some of the epitopes of a particular antigen molecule are likely to be free, whereas others are in the bound state. So-called bound antigen molecules may, in fact, possess between 0 and $s - 1$ free epitopes. Accordingly, it is the concentration of free antibody d that is measured experimentally after free and bound antibody have been separated by procedures such as ultrafiltration or centrifugation, or by simple washing when the multivalent antigen is immobilized in a solid-phase assay. The value of d is then used to calculate $y = B - d$ and f.

When the experimental protocol consists in measuring free antibody instead of free antigen, it is nevertheless possible to represent the data by means of Eq. (3). After deriving the value of the antigen valence from a plot of Eq. (4), and since $ny = sx$, it is possible to calculate $r = ny/sB$ and to construct r/c versus sr plots (Hardie and Van Regenmortel, 1975).

One oddity of this representation is that linear plots are always obtained with large multivalent antigens, even when the antibody preparation that is used is highly heterogeneous, as is normally the case with polyclonal antiserum (Hardie and Van Regenmortel, 1975). The linear appearance of such plots has led some authors to the mistaken conclusion that their antibody preparations were homogeneous relative to the affinity constant (Mamet-Bratley, 1966; Anderer et al., 1971; Day, 1990).

Another commonly used transformation of the mass action equation is obtained by dividing the numerator and denominator of Eq. (2) by A:

$$K = \frac{y/a}{(s - ny/A)(B - y)} = \frac{f}{(s - nf)d}$$

and

$$\frac{f}{d} K(s - nf) \tag{4}$$

This transformation allows the construction of f/d versus nf plots from which the value of K and of the antigen valence s can be derived (Rappaport, 1959; Day, 1990; Hardie and Van Regenmortel, 1975). In such a plot (see Fig. 1b), when $f/d \rightarrow 0$, $s - nf \rightarrow 0$, and $nf \rightarrow$ s. When monovalent Fab fragments of antibody molecules are used in such measurements, the value of s can be derived in a straightforward manner by extrapolation. It is usually assumed that antigenic valency is a constant molecular parameter and not a variable influenced by the ratio of antibody to antigen (Day, 1990). The antigenic valency then corresponds to the maximum number of antibody molecules that can be bound simultaneously (Van Regenmortel, 1988). For instance, in the example of tobacco mosaic virus, which contains 2130 identical protein subunits, steric hindrance is responsible for the observation that the antigenic valency is 800 and not 2130, as has sometimes been assumed (Anderer et al., 1971; Hardie and Van Regenmortel, 1975). Cowan and Underwood (1988) have developed a theory that accounts for the binding of antibody to a multivalent antigen under conditions of steric hindrance. Their theory predicts that the steric hindrance effect necessarily leads to Scatchard plots that are curved, not linear, also for homogeneous binding of monoclonal antibodies. In a recent analysis of binding data obtained in the tobacco mosaic virus system, experimental points fit a quadratic curve better than a straight-line Schatchard plot (Pellequer and Van Regenmortel, 1993a), a finding that agrees with the model proposed by Cowan and Underwood (1988). However, the error in the determination of K made when the points are fitted to a straight-line, instead of a curve, is not large.

For bivalent antibody molecules, plots of f/d versus nf are often biphasic, corresponding to regions where the antibody binds in a monovalent and bivalent manner, respectively (Van Regenmortel and Hardie, 1976). Near antibody saturation, the molecules bind monovalently and the slope of the plot gives a K value that is considerably smaller (usually by about two orders of magnitude) than in the region of antigen excess when the same antibody binds bivalently. When the antibody binds to virus particles or cell surfaces presenting a large number of identical epitopes, the two Fab arms of an IgG molecule will bind to neighboring epitopes on the same particle, a situation known as monogamous bivalent binding, characterized by a high functional avidity (Day, 1990; Kaufman and Jain, 1992).

For pentameric IgM molecules, the multiplicity of attachment sites further widens the difference between the "intrinsic affinity" of a single paratope and the "functional affinity" of the molecule as a whole (Hornick and Karush, 1972).

When in a binding test the antigen concentration is kept constant while the antibody concentration is varied and the amount of free antibody is determined experimentally, it is possible to represent the binding data by the following transformations of the mass action relationship (Fazekas de St. Groth, 1979):

$$\frac{ny}{nd} = K(As - ny) \tag{5}$$

$$ny = -\frac{1}{K}\left(\frac{ny}{nd}\right) + As \tag{6}$$

$$\frac{1}{ny} = \frac{1}{KAs}\left(\frac{1}{nd}\right) + \frac{1}{As} \tag{7}$$

Table 1 Calculation of Antibody Affinity Constant K and of Total Antigenic Sites As From Binding Data Obtained in Solid-Phase Immunoassay and Presented According to Eqs. (5)–(16)

Equation	5	6	7	8	9	10	11	12	13	14	15	16
Slope	$-K$	$-1/K$	$1/K{\cdot}As$	$K{\cdot}As$	As	$1/As$	$-K$	$-1/K$	$1/K{\cdot}Bn$	$K{\cdot}Bn$	Bn	$1/Bn$
Abscissa at origin	As	$K{\cdot}As$	$-K$	$1/As$	$1/K{\cdot}As$	$-1/K$	Bn	$K{\cdot}Bn$	$-K$	$1/Bn$	$1/K{\cdot}Bn$	$-1/K$
Ordinate at origin	$K{\cdot}As$	As	$1/As$	$-K$	$-1/K$	$1/K{\cdot}As$	$K{\cdot}Bn$	Bn	$1/Bn$	$-K$	$-1/K$	$1/K{\cdot}Bn$

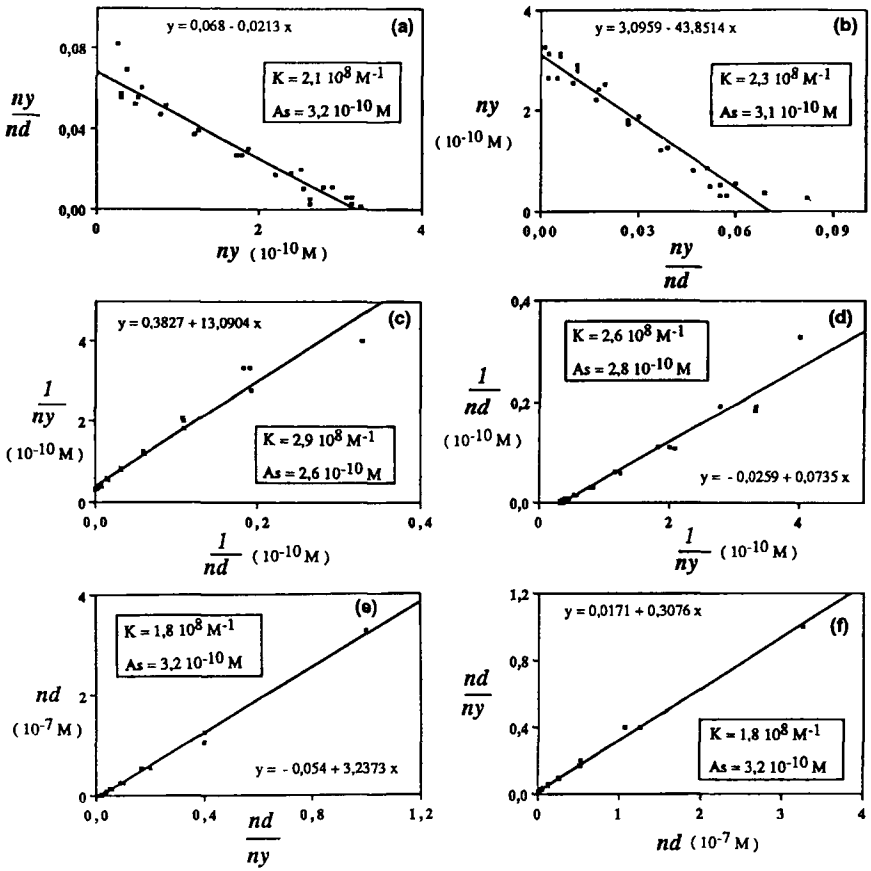

Figure 2 (a)–(f) Experimental data of Schots et al. (1988) plotted in the form of Eq. (5)–(10), respectively. ny = bound antibody sites (mol/L); nd = free antibody sites (mol/L). The values of K and As (total antigen sites) were obtained from the slope or by extrapolation as indicated in Table 1. (From Azimzadeh and Van Regenmortel, 1990.)

$$\frac{1}{nd} = KAs\left(\frac{1}{ny}\right) - K \tag{8}$$

$$nd = As\left(\frac{nd}{ny}\right) - \frac{1}{K} \tag{9}$$

$$\frac{nd}{ny} = \frac{1}{As}(nd) + \frac{1}{KAs} \tag{10}$$

These various representations make it possible to calculate K and As by extrapolation or from the slope of the plot as indicated in Table 1. Plots of such calculations for binding data obtained in a solid-phase immunoassay are presented in Fig. 2.

When, in a binding test, the antibody concentration is kept constant, while the antigen concentration is varied and the amount of free antigen is determined experimentally, the data can be represented as follows:

$$\frac{sx}{sc} = K(Bn - sx) \tag{11}$$

$$sx = -\frac{1}{K}\left(\frac{sx}{sc}\right) + Bn \tag{12}$$

$$\frac{1}{sx} = \frac{1}{KBn}\left(\frac{1}{sc}\right) + \frac{1}{Bn} \tag{13}$$

$$\frac{1}{sc} = K \cdot Bn\left(\frac{1}{sx}\right) - K \tag{14}$$

$$sc = Bn\left(\frac{sc}{sx}\right) - \frac{1}{K} \tag{15}$$

$$\frac{sc}{sx} = \frac{1}{Bn}(sc) + \frac{1}{KBn} \tag{16}$$

These representations make it possible to calculate K and Bn as indicated in Table 1.

III. EXPERIMENTAL APPROACHES

A. Solution-Phase Assays

In these assays, the interaction between antigen and antibody as well as the separation of free molecules from the complex occurs in solution.

To determine the affinity constant of an antibody directed to a small monovalent hapten, it is customary to experimentally measure the concentration c of free antigen after having separated the small hapten molecules from the larger antigen–antibody complex, for instance by equilibrium dialysis (Eisen, 1964a; Giles et al., 1983; Pascual and Clem, 1988). The amount of bound antigen is calculated as $x = A - c$, and the results are plotted according to Eq. (3). The value of K can be obtained without prior knowledge of the total concentration of antibody sites Bn, since it is possible to derive the value of Bn from binding measurements on serial dilutions of an uncalibrated antibody preparation. This can be done by using one of Eqs. (11)–(16). Equation (13), which is referred to as a Langmuir plot because the form of the equation resembles that of a Langmuir adsorption isotherm, has often been used for this type of calculation (Stewart and Petty, 1972a,b). In this plot, when $1/sc \rightarrow 0$, $1/sx \rightarrow 1/Bn$ and it is thus possible to derive the value of Bn by extrapolation.

For large multivalent antigens, it is the concentration of free antibody d that is measured in the experiment, after free and bound antibody have been separated by procedures such as ultrafiltration or ultracentrifugation. This value of d is used to calculate the amount of bound antibody y, and the value of K is obtained from f/d versus nf plots.

When sufficient quantities of the reagents are available and the antigen is much larger than the antibody, free antibody separated from the complex by ultracentrifugation can be measured spectrophotometrically (Hardie and Van Regenmortel, 1975).

Data obtained in this manner are illustrated in Fig. 3, which shows two f/d versus f plots of the binding of tobacco mosaic virus (TMV) to specific rabbit IgG and Fab fragments, respectively. The binding data used to construct these plots were obtained as follows. A series of dilutions of a virus preparation (1 ml) were mixed with a constant concentration of specific antibody. After ultracentrifugation, the amount of free antibody (d) in the supernatant was determined, and the

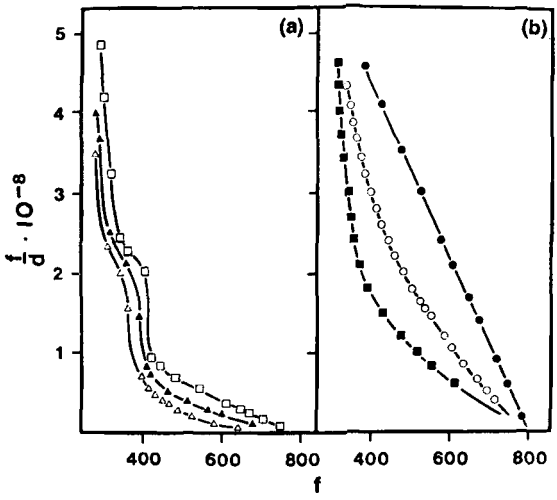

Figure 3 Determination of the avidity of tobacco mosaic virus (TMV) antibodies. (a) Plots of f/d vs f representing the interaction between BMV and specific IgG used at 6 mg/ml (□), 1.8 mg/ml (▲), and 0.6 mg/ml (△). The curves extrapolate to the antigenic valence of TMV, $s = 780$. (b) Plots of f/d vs f representing the interaction between TMV and specific Fab fragments used at 1.9 mg/ml (●), 1.18 mg/ml (○), and 0.6 mg/ml (■). The antigenic valence is $s = 800$. (From Van Regenmortel, 1982.)

amount of bound antibody was calculated from $y = B - d$. The plots obtained with different concentration of Fab extrapolate to $s = 800$, which is the antigen valence of the virus. The plots obtained with IgG extrapolate to $s = 780$ (see Fig. 3). The similarity in the two s values indicates that in antibody excess, the IgG molecules bind univalently. The biphasic nature of the plot in Fig. 3a indicates that bivalent binding of IgG ($n = 2$) occurs at lower antibody/antigen ratios when $f < 400$. The higher slope in this part of the plot corresponds to the higher avidity of the antibodies when they bind in a bivalent manner (Day, 1990; Van Regenmortel and Hardie, 1976). This interpretation of the biphasic plot is supported by the data shown in Fig. 4, which indicate that small successive additions of antibody to a given amount of virus results in the binding of fewer antibody molecules per virus particle than a single addition of the total amount of antibody. Adding small amounts of antibody to the virus favors bivalent binding, which doubles the number of antigenic sites covered, without increasing the number of antibody molecules bound. In this example, the antigen valence is obtained by multiplying the limiting value of f by 2, which again leads to $s = 800$.

When only small amounts of reagents are available, it is not possible to use spectrophotometric measurements to evaluate d. Here, the amount of free antibody can be measured by enzyme-linked immunosorbent assay (ELISA) after separating free antibody from the complexes by centrifugation in bench-top instruments, such as the Beckman Airfuge or the Beckman TL-100 ultracentrifuge (Bator and Reading, 1989). This is illustrated in a recent study of the reaction of TMV with a monoclonal antibody (Azimzadeh and Van Regenmortel, 1991). In this method no labeling either of antibody or antigen is required, and any alteration of their immunochemical reactivity because of chemical coupling procedures is avoided. The amount of free monoclonal antibody (MAb) in the supernatant after centrifugation was calculated from a calibration plot of

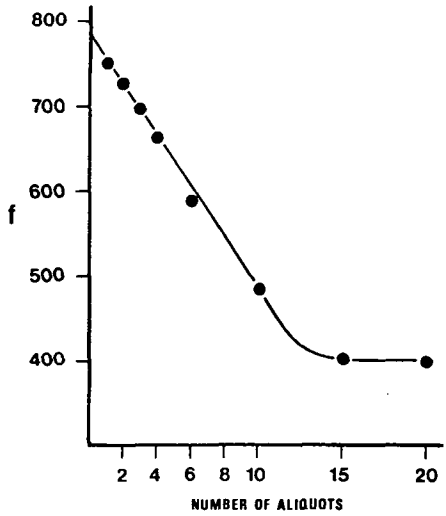

Figure 4 Variation in f observed when the same total quantity of specific IgG antibody (3.6 mg) is added to 1 mg TMV but in different aliquots. With small successive additions of IgG, the plot extrapolates to $f = 400$. Assuming monogamous bivalent binding of IgG, this corresponds to an antigenic valence of TMV of $s = 800$. When all the antibody is added at once, the antigenic valence of TMV obtained by extrapolation on the y-axis is $s = 780$. (From Van Regenmortel, 1982.)

antibody concentration versus absorbance of 405 nm constructed from sandwich ELISA experiments. Plots of absorbance versus log of antibody concentration were linear between 10 and 50 ng/ml antibody. Supernatants were diluted to be in the predetermined linear range of the plot.

As shown in Fig. 5b, this method made it possible to obtain data both in the zone of antigen excess, where antibodies bind primarily in a bivalent manner, and in the zone of antibody excess, where monovalent antibody binding occurs. However, it was necessary to correct for the very small amount (about 0.6%) of inactive molecules in the MAb preparation. Such unreactive antibody molecules can lead to large errors in d values. In the absence of any correction, f/d versus f plots with a positive slope are obtained, from which it is impossible to calculate a meaningful value of K in the region of bivalent binding (see Fig. 5a). The proportion of inactive antibody molecules present in the assay can be assessed by the extent to which the value of Bn, derived from Eq. (15) and (16), differs from the amount of antibody supposedly introduced in the test. The amount of inactive antibody is then subtracted from experimental d values. A theoretical simulation of the influence of different levels of inactive antibody on the appearance of f/d versus f plots showed that small amounts of inactive antibody play an increasingly important role the higher the value of the affinity constant (Azimzadeh and Van Regenmortel, 1991). This is to be expected, since errors in d will be magnified when K is large and the amount of free antibody concomitantly smaller.

When the size of the antigen is similar to that of an antibody molecule, it is not possible to completely separate free from bound antibody by sedimentation. In this situation, another approach, based on equilibrium sedimentation, can be used that allows the concentration of labeled free antibody to be measured at the meniscus of the centrifuge tube (Jackson et al., 1983).

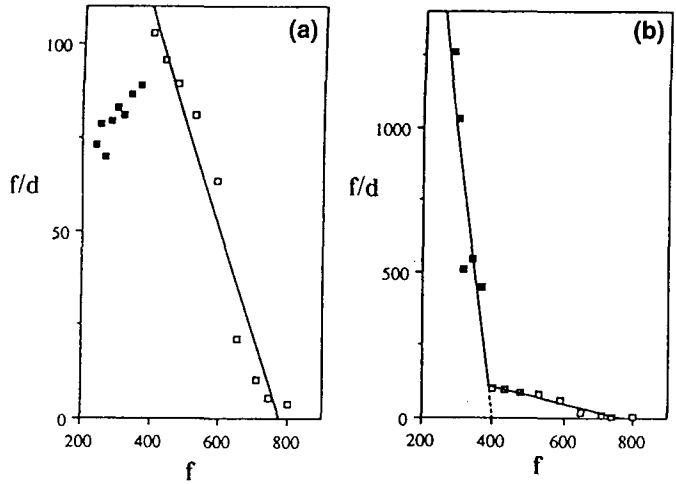

Figure 5 TMV–MAb 253P binding data plotted according to Eq. (4). Open and black symbols correspond, respectively, to monovalent and bivalent binding of IgG molecules. Bivalent binding has been represented before (a) and after (b) correcting for the presence of 0.55% inactive antibody. (From Azimzadeh and Van Regenmortel, 1991.)

For instance, when Airfuge tubes containing the antigen and Fab fragments are centrifuged at 33,000 rpm for 20 h, free Fab molecules will become uniformly distributed throughout the tube, whereas bound Fab will tend to form a stable concentration gradient from the top to the bottom of the tube. The contents of the tube are fractionated, and the concentration of the reagents in each fraction is measured. By choosing centrifugation conditions under which the bound Fab fragments are selectively removed from the meniscus, it is possible to determine the concentration of free Fab at this position and thereby to calculate K (Jackson et al., 1983). One advantage of the method is that the relative molecular masses of the two interacting components can be measured at the same time. Recently, this method was used to measure the affinity constant of the antilysozyme D1.3 MAb, leading to a value of $K = 1.6 \cdot 10^9/M^{-1}$ (McInerney et al., 1993).

When labeled antigen molecules are used, various procedures can be applied to separate free labeled antigen from bound complexes; for instance, precipitation of antibody with 50% ammonium sulfate (Kim et al., 1975; Parrat et al., 1982), with 15% polyethylene glycol (Hetherington, 1988), or by binding to protein A (Brunda et al., 1977) or to anti-immunoglobulin antibody (Stewart and Petty, 1972a; Glass et al., 1973). Precipitation conditions need to be controlled carefully, since the equilibrium may be disturbed (Seppälä, 1975), antigen molecules may be precipitated together with the complexes (Berzofsky, 1984), and not all antibody subclasses may be equally precipitable (Wagener et al., 1983). When the antibody is biotinylated, it can be precipitated by the addition of avidin (Clark and Todd, 1982). Alternatively, the antigen may be removed from solution, for instance, by adsorption to charcoal (Herbert et al., 1965; Collignon et al., 1988) or, when it is biotinylated, by precipitation with avidin (Krüger et al., 1989). Antigen–antibody interaction can also be measured by various spectrofluorometric methods (Parker, 1978), such as fluorescence quenching (Eisen, 1964b), fluorescence polarization (Friguet et al., 1989), or enhanced fluorescence of the bound ligand (Karush and Tang, 1988). An

overview of the different methods for measuring antibody affinity has been presented by Stewart (1986). Methods based on the determination of the kinetic rates of association and dissociation are discussed in Chapter 36, as well as in several recent reports (Mason and Williams, 1986; Malmborg et al., 1992; Zeder-Lutz et al., 1993; Pellequer and Van Regenmortel, 1993b).

B. Solid-Phase Assays

In solid-phase assays, a constant amount of antigen is usually adsorbed to plastic, but its total concentration remains unknown. Varying amounts of antibody are added to the microtiter wells and, after removing free antibody by washing, the amount of bound antibody is measured experimentally.

The binding data can be represented by Eqs. (5)–(10). Examples of such plots based on the experimental results of Schots et al. (1988) with a peroxidase-labeled MAb are shown in Fig. 2. As discussed by Fazekas de St. Groth (1979) the greatest precision in these plots is provided by intercepts with minimal extrapolation and slopes close to unity.

The most even distribution of points and most reliable estimates of *As* and *K* were obtained from the plots of Eqs. (5) and (6), which are inverse functions of each other.

One of the advantages of the method described by Schots et al. (1988) is that it uses only small quantities of reagents; a disadvantage is that it requires a highly purified, peroxidase-labeled preparation of monoclonal antibody.

When the total concentration of antibody sites (*Bn*) is unknown, it may be useful to express Eqs. (5)–10 not in terms of absolute concentrations of bound (*ny*) and free sites (*nd*), but in terms of the fraction of antibody bound (α) or free ($1 - \alpha$). The value of α lies between 0 and 1 and corresponds to *ny*/*Bn*. Experimentally it is possible to measure either α or $1 - \alpha$.

As shown by Fazekas de St. Groth (1979), Eq. (7) expressed in terms of α and $1 - \alpha$ becomes:

$$\frac{1}{\alpha} = \frac{Bn}{As} - \frac{1}{KAs}\left(\frac{1}{1-\alpha}\right)$$

By plotting $1/\alpha$ versus $1/1 - \alpha$, it is possible to obtain the value $1/KAs$ from the slope. *K* can then be calculated without knowing the value of *Bn* (Fazekas de St. Groth, 1979).

Many authors use a type of assay format in which antigen is directly adsorbed to the solid-phase. Because proteins usually undergo some denaturation when they are adsorbed to plastic (Soderquist and Walton, 1980; Friguet et al., 1984; Darst et al., 1988) it is questionable whether the value of *K* that is measured in this way pertains to the interaction with an epitope in its native state. For this reason it is preferable to capture the antigen on the plastic by a first layer of adsorbed antibodies and to measure *K* by a double-antibody sandwich assay format. When the antigen is trapped on the plastic by a first layer of antibodies, its immunoreactivity tends to be preserved (Butler, 1992).

Frankel and Gerhard (1979) have used a solid-phase radioimmunoassay for measuring the affinity of monoclonal antibodies to influenza virus. The virus was adsorbed to the plastic, and bound antibodies were titrated by ^{125}I-labeled rabbit antimouse $(Fab')_2$ antibody using a standard curve established with known quantities of the viral antibody. Because the concentration of antigen was kept constant, it was not necessary to know its actual concentration to calculate *K* according to Eq. (5). The total amount of antibody sites (*Bn*) present at each antibody dilution was titrated in separate wells containing up to a 25-fold excess of antigen. The authors attempted to evaluate the antigenic valency of the virus by extrapolation, using Eq. (5), assuming that half the viral surface was exposed to solvent, and found an approximate value of $s = 1000–2700$. Various influenza monoclonal antibodies were found to have *K* values of 4.4×10^8–$7.0 \times 10^9\ M^{-1}$.

The method described by Frankel and Gerhard (1979) has been used for measuring the

affinity of monoclonal antibodies to influenza virus (Breschkin et al., 1981; Lubeck and Gerhard, 1982; Wang et al., 1986), to human prothrombin (Lewis et al., 1983), and to cystatin C (Ishiguro et al., 1989), as well as for estimating the affinity of polyclonal antibodies to estradiol (Latif and Mandal, 1987) and meningococcal polysaccharide (Mandrell and Zollinger, 1982). Several modifications of the Frankel and Gerhard (1979) procedure have been introduced. Instead of ^{125}I-labeled antimouse antibodies, Kirkland et al. (1988) used biotinylated antibodies that were detected with iodinated streptavidin. Aasted and Bloom (1984) adsorbed the antigen on polystyrene beads that were incubated in ELISA plates and detected bound antibody by means of iodinated protein A. Alsheikhly et al. (1985) fixed the antigen on cellulose disks activated with cyanogen bromide, and measured the binding of ^{125}I-labeled monoclonal antibodies in a test tube.

Difficulties that may arise when antibodies labeled with an enzyme or with ^{125}I are used have been discussed by Azimzadeh and Van Regenmortel (1990). During the labeling procedure, the antibody-binding capacity tends to be affected in a detrimental manner, resulting in lower K values than with unlabeled antibody.

On the other hand, when labeled anti-immunoglobulin reagents are used for titrating unlabeled antibody, it is important to ascertain that the labeled reagent reacts equally well with all immunoglobulin subclasses. If this is not the case, the labeled reagent should be calibrated with a standard globulin of the same subclass as that of the antibody to be tested.

C. Enzyme-Linked Immunosorbent Assay Equilibrium Titration Method

Friguet et al. (1985) developed a method that uses a solid-phase assay for measuring the amount of free antibody present at equilibrium in a solution-phase antigen–antibody reaction mixture. The solution-phase mixture is incubated in antigen-coated wells using conditions under which only about 10% of the free antibody is captured. This ensures that the equilibrium is not disturbed. The proportion of free antibody is calculated from the difference in absorbance measured in the presence and absence of antigen, read from the linear portion of a calibration plot of absorbance versus antibody concentration. In conditions under which a linear dependence of optical density (OD) versus antibody concentration is observed in the ELISA assay, and provided that the total antibody concentration is known, free-antibody concentration at equilibrium can be determined using the relation:

$$\frac{nd}{Bn} = \frac{OD_d}{OD_B}$$

where OD_d is the optical density measured in the presence of a given concentration of antigen, and OD_B is the optical density measured in the absence of antigen. The fraction of bound antibody α can be defined by:

$$\alpha = \frac{OD_B - OD_d}{OD_B}$$

However, this is true only if no readjustment of the equilibrium in the liquid-phase occurs during the incubation of the mixture in the coated wells (Friguet et al., 1989). This condition can be verified by establishing that, under the experimental conditions used (for a given quantity of antigen coated in the wells and incubation time), a maximum amount of about 10% free antibody is trapped. For that purpose, after incubating the antibody at various known concentrations in the coated wells for an appropriate time, the contents of each well are transferred to another plate coated in the same conditions and incubated for the same time. The difference in OD values

obtained for the different antibody concentrations before and after transfer should not exceed 10%.

The binding data are presented according to

$$\frac{ny}{sc} = K(Bn - ny) \tag{17}$$

Using the fraction of bound antibodies (α), the quantities ny and sc can be expressed as

$$ny = \alpha Bn$$

$$sc = As - Bn$$

Equation (17) then becomes

$$\frac{\alpha}{sc} = K(1 - \alpha) \tag{18}$$

Equation (18) allows the construction of Scatchard plots of the type α/sc versus α, from which the value of K can be obtained from the slope. The extrapolation on the x-axis corresponds to the value unity. An example is given in Fig. 6a.

If the concentration of total binding sites (Bn) is unknown, it is possible to obtain K according to the Klotz expression of the mass action law (Klotz, 1953):

$$\frac{1}{\alpha} = \frac{1}{K} \cdot \frac{1}{(As - \alpha Bn)} + 1 \tag{19}$$

where the quantity sc is expressed as

$$sc = As - \alpha Bn$$

When the total concentration of antigen that is used is much larger than that of the antibody ($As > 10 \times Bn$), Bn and αBn are negligible compared with As, and sc can be approximated by As.

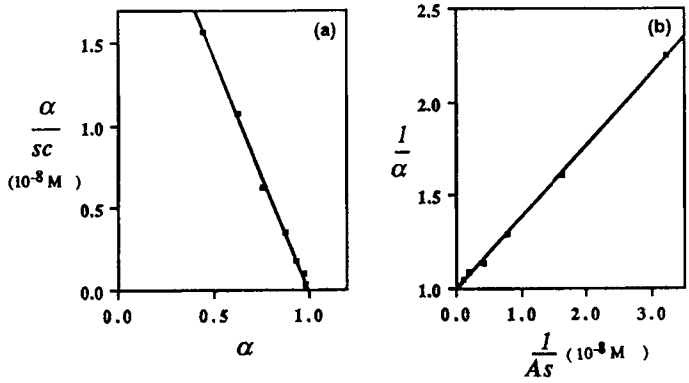

Figure 6 Experimental data of Friguet et al. (1985) for the binding of an MAb to the β_2-subunit of *Escherichia coli* tryptophan synthase plotted according to (a) Eq. (18) and (b) Eq. (20). α = fraction of bound antibody sites; sc = free antigen sites (mol/L); As = total antigen sites (molL). In (a), the slope gives the value of $-K$. In (b), the slope gives the value of $1/K$. (From Azimzadeh and Van Regenmortel, 1990.)

Table 2 Values of Antibody Affinity Constants Obtained by Various Techniques

Technique	Antigen	Antibody	K (M^{-1})	Ref.
Equilibrium dialysis	[^{3}H]DNP	IgG, IgG2a, IgG2b	0.4×10^6–4.1×10^7	Steward and Lew (1985)
	[^{125}I]NIP	3 IgE MAbs	2.6×10^6–2.1×10^7	Bose et al. (1986)
	[^{3}H]DNP	8 IgM MAbs	4.0×10^4–1.2×10^7	Pascual and Clem (1988)
Fluorescence	Fluorescein	4-4-20	1.8×10^{11}	Kranz and Voss (1981)
	Fluorescein	4-4-20	3.4×10^{10} (aqueous buffer) 3.2×10^7 (50% 2-methyl-2-4-pentanediol)	Gibson et al. (1988)
	Fluorescein	5 IgG1, IgG2 MAbs	9.9×10^7–1.8×10^{10}	Kranz et al. (1982)
	PC	8 IgG1, IgG3 MAbs	1–4.4×10^5	Rodwell et al. (1983)
	DPPC		4.8×10^5–2.6×10^6	
	PC/DPPC	1 IgA MAbs	$4.3/4.8 \times 10^5$	
	PC	5 IgM MAbs	2.4–3.2×10^5	
	DPPC		1.2–5.7×10^5	
	Ars	10 IgG1, IgG2 MAbs	0.98×10^5–4.5×10^7	Rothstein and Gefter (1983)
	Ars	2 IgG MAbs	1.3–1.7×10^6	
	Fluorescein	5 IgG1, IgG2, IgG3 MAbs	3.3×10^7–1.7×10^{10}	Bates et al. (1985)
	Fluorescent peptide	3 IgG MAbs	1.4×10^5–2.1×10^6	Karush and Tang (1988)
	Fluorescent peptide	2 IgM MAbs	2–2.7×10^4	
	Oxazolone	20 IgG MAbs	1.1×10^6–$>5 \times 10^8$	Gherardi et al. (1990)
	Lysozyme	D1.3	2.7×10^8	Foote and Winter (1992)

Precipitation	[^{125}I]human IgG	6 IgG MAbs	1.9×10^6–5.5×10^8	Jacobsen et al. (1982)
	[^{3}H]interleukin-2	3 IgG1, IgG2	8.3×10^6–3.2×10^7 (37°C)	Budd and Smith (1986)
	[^{3}H]interleukin-2	3 IgG1, IgG2	5.3×10^7–7.1×10^7 (4°C)	
	[^{3}H]interleukin-2	I IgM MAbs	3.4×10^7 (37°C)	
	[^{3}H]interleukin-2	1 IgM MAbs	1.7×10^8 (4°C)	
	[^{3}H]digitoxin	9 IgG1, IgG2, IgG3 MAbs	8.0×10^8–2.5×10^{10}	Collignon et al. (1988)
	[^{3}H]digoxin	3 IgG1 MAbs	9.2×10^6–5.4×10^9	Panka et al. (1988)
ELISA titration equilibrium method	Tryptophan synthetase β^2 subunit	IgG MAb	2.8×10^8	Friguet et al. (1985)
	13 peptides	MAb 164-2	6.7×10^6–1.0×10^9	Larvor et al. (1991)
	Polyreactive	Human MAbs	10^3–10^7	Nakamura et al. (1988)
	Monoreactive	Human MAbs	10^9–10^{11}	
	Methotrexate	23 MAbs	3.5×10^5–7.0×10^9	Cot et al. (1987)
	RNA polymerase II subunits	3 IgM MAbs	1.3–2.5×10^6	Dahmus et al. (1988)
	RNA polymerase II subunits	G_7A_5 MAb	1.0×10^8	
	Lysozyme	D1.3 Fab	4.5×10^7	Amit et al. (1986); Harper et al. (1987)
Biosensor (BIAcore)	Lysozyme	D1.3	4.0×10^9	Chitarra et al. (1993)
Sedimentation equilibrium	Lysozyme	D1.3	1.6×10^9	McInerney et al. (1993)

In such a situation, Eq. (19) can be simplified to

$$\frac{1}{\alpha} = \frac{1}{(KAs)} + 1 \tag{20}$$

A plot of $1/\alpha$ versus $1/As$ will have a slope of $1/K$, whereas the extrapolation on the y-axis will give a value of unity. An example is given in Fig. 6b.

The method of Friguet et al. (1985) offers several advantages over other procedures. It requires no labeling of antigen or antibody and thereby avoids the changes in immunoreactivity that often accompany labeling. It requires very small quantities of reagents and measures K in solution-phase equilibrium conditions. It thus avoids the ambiguities linked to measuring antibody affinity for an antigen that has been altered by adsorption to a solid-phase.

The method of Friguet et al. (1985) has been widely used, for instance, for measuring the affinity of autoantibodies (Ternynck and Avrameas, 1986), of 7S fragments of IgM rheumatoid factors (Robbins et al., 1988), and of various murine and human monoclonal antibodies (Dahmus et al., 1988; Nakamura et al., 1988).

The original procedure described by Friguet et al. (1985) did not consider that an IgG molecule can be bound to a solid-phase antigen by one or by two binding sites. As discussed by Stevens (1987), the fraction of apparent "free" IgG is not the same as the fractional free binding sites. Since only double-liganded IgG is scored as bound when it is assayed on the solid-phase antigen, the extent of antigen binding is underestimated. On the basis of binomial analysis, Stevens (1987) showed that it is possible to convert apparent concentrations of bound IgG to actual concentrations of liganded binding sites and thereby to calculate valid affinity constants for intact IgG. In the absence of this correction, the affinity constant is underestimated by a factor of at least 2. At very high saturation of antibody by antigen, this type of error is minimized (Friguet et al., 1989).

A number of K values for different antibodies measured by various techniques are listed in Table 2.

IV. COMPARISON OF THE DIFFERENT METHODS

A comparison of the three approaches used for calculating K values is presented in Table 3. In the liquid-phase and the ELISA equilibrium titration methods, the equilibrium between antibody and antigen occurs in solution, whereas in the solid-phase method, one of the reactants in solution is in equilibrium with the other one immobilized on the solid-phase. Whereas the solution-phase method is less sensitive than the other two methods, it has the advantages that it allows the antigen valence to be calculated and that K values can be obtained for both monovalent and bivalent binding of antibodies. However, frequently, the assay conditions are such that only bivalent

Table 3 Comparison Between Different Assays Used to Measure Antibody Affinity

Parameter	Solution-phase assay	Solid-phase assay	ELISA equilibrium titration method
Sensitivity	μg/ml	ng/ml	ng/ml
Monovalent antibody binding	+	+	−
Bivalent antibody binding	+	+	+
Antigenic valence obtained	+	−	−
Interference by inactive antibodies	+	−	−
Knowledge of antigen concentration necessary	+	−	+

binding of antibodies is observed (Frankel and Gerhard, 1979; Schots et al., 1988; Azimzadeh et al., 1992b). To make valid comparisons between the affinities of different antibodies, it is important to ascertain whether monovalent or bivalent binding occurs. This is also important in the ELISA equilibrium titration method, since K values will be underestimated if all the IgG molecules are not bound in a bivalent manner (Stevens, 1987). Since an antibody molecule bound in a monovalent manner can still bind by its second paratope to the solid-phase antigen, it could be scored as a free molecule, even though it has interacted with the antigen by one of its combining sites (Friguet et al., 1989).

A knowledge of the antigen concentration is not necessary for the solid-phase method, since the amount of antigen adsorbed to the solid-phase is constant, but it is required with the other two methods. For multivalent antigens, the antigen valence can be determined only by the liquid-phase method. It is thus necessary to determine the valency by this method before the ELISA equilibrium titration method can be used with multivalent antigens.

Interference of inactive antibody molecules in the measurement of free antibody is especially prevalent in the liquid-phase method and is more pronounced, the higher the affinity of the antibody (Azimzadeh and Van Regenmortel, 1991).

When the affinity of an antibody is measured in both liquid-phase and solid-phase assays, considerable differences in K values are commonly observed. For instance, Underwood (1985) reported lower K values when the affinity of nine MAbs was measured by a solid-phase assay, although in general solid-phase assays have been reported to give higher K values than liquid-phase assays (Lehtonen, 1981; Nygren et al., 1986; Azimzadeh et al., 1992b).

These differences between liquid-phase and solid-phase assays can be explained by differences in kinetic rates. It seems that both association and dissociation rates are lower in solid-phase tests (Mason and Williams, 1980; Li, 1985; Nygren et al., 1986) than in liquid-phase tests (Karush, 1978; Steward, 1977; Friguet et al., 1989), and that the dissociation rate can be decreased by as much as two orders of magnitude. Dissociation rates are sometimes so low that reactions can be considered as practically irreversible (Mason and Williams, 1980; Stenberg and Nygren, 1982). This effect arises from the very high effective local epitope density at the solid surface, compared with the concentration if the same number of sites were distributed in bulk solution. In these conditions, there is a high probability of reassociation with the surface instead of diffusion transport away from the surface after dissociation. It has also been shown that the equilibrium may not be reached within the usual incubation time in ELISA (Nygren et al., 1987; Stenberg and Nygren, 1988), and it may be beneficial to stir the plates during incubation (Mushens and Scott, 1990). Effectively, the diffusion limited at a plane surface is no longer proportional to time, but rather to the square root of time. These observations suggest that solid-phase data should not be interpreted on the assumption that conditions of solution equilibrium are fulfilled. It should come as no surprise, therefore, that K values measured by solid-phase and liquid-phase methods usually differ significantly. On the other hand, the ELISA equilibrium titration method, which combines some of the advantages of both solid-phase and liquid-phase techniques, gives K values that agree closely with values obtained by liquid-phase assays (Azimzadeh et al., 1992b; Pellequer and Van Regenmortel, 1993b).

For polyclonal antibodies, several authors have reported a dependence of K values on antibody concentration (Hudson, 1968; Larralde and Farber, 1972; Arend and Mannick, 1974; Varitek and Day, 1979). This phenomenon can be explained by the heterogeneity of the antibody population. At high antibody concentration, antibodies of low affinity are able to bind, whereas at low antibody concentration, those of high affinity bind preferentially. To make valid comparisons between antisera, it is necessary to work at a constant antigen–antibody ratio (Kim et al., 1975; Van Regenmortel and Hardie, 1979).

For MAbs, a dependence of K values on antibody concentration is not expected. However,

in a recent study of the affinity of MAbs directed to tobacco mosaic virus, it was shown that the apparent affinity constant decreased about 25-fold when the antibody concentration used in the assay was increased from 30 ng/ml to 35 μg/ml (Azimzadeh et al., 1992a). This phenomenon was found to be caused by a decrease in kinetic association rate constant where antibody concentration increased and the antigen surface became saturated (Pellequer and Van Regenmortel, 1993b). In principle, the most reliable K values should be those obtained with the lowest antibody concentrations (Jacobsen et al., 1982; Steensgaard et al., 1980). It is likely that steric hindrance, which is prevalent with a viral antigen such as tobacco mosaic virus that possesses over 2000 identical subunits of 17.5 kDa, also contributed to the observed concentration dependence of K values.

Differences observed between K values measured by different methods underline the operational limitations inherent in the various techniques. Nevertheless, valid comparisons between the affinity of different antibodies is possible when a single technique is used under standardized conditions.

V. THERMODYNAMICS

The affinity constant, K, is directly related to the free energy change, ΔG, of the antigen–antibody reaction by the equation

$$\Delta G = -RT \ln K \tag{21}$$

where R is the gas constant (1.98 cal or 8.31 J/K per mole), and T is the absolute temperature in kelvin. The standard free-energy change ΔG^0 is defined as the ΔG pertaining to standard conditions (i.e., when unit molar concentrations of the reactants interact to form 1 mol of complex):

$$\Delta G^0 = -RT \ln K^0 \tag{22}$$

When the binding energy of single antigen and antibody molecules is considered, the gas constant is replaced by the Boltzmann constant $k = R/N$

$$\Delta G^0 = -kT \ln K^0 \tag{23}$$

From Eq. (22), it follows that a tenfold increase in binding affinity corresponds to a free-energy change of only 1.4 kcal/mol at 25°C (298 K). This means for instance that K values of $10^6\ M^{-1}$, $10^9\ M^{-1}$, and $10^{12}\ M^{-1}$ correspond to a ΔG of 8.4, 12.6, and 16.8 kcal/mol, respectively. The entire range of affinity constants normally encountered in antigen–antibody interactions, therefore, encompass no more than 8 kcal of free-energy change (see Table 2, Chapter 23).

Since the energy of a single hydrogen bond is about 4.5 kcal/mol (or only about 1 kcal/mol when the loss of hydrogen bonds with water occurring during the formation of the complex is taken into account), it follows that a small modification in the antigen resulting in the loss of a few hydrogen bonds with the antibody will lead to considerable changes in affinity (see Chapter 23).

The free-energy change is composed of an enthalpic and an entropic term:

$$\Delta G = \Delta H - T\Delta S \tag{24}$$

where ΔH is the change in enthalpy (the heat of the reaction) and ΔS is the entropy (a term expressing the disorder produced by the reaction).

When K is measured at different temperatures, the enthalpy can be calculated from:

$$\frac{d \ln K}{\mathrm{dT}} = \frac{\Delta \mathrm{H}}{RT^2} \tag{25}$$

or

$$\ln \frac{K_1}{K_2} = \frac{\Delta H}{R} \frac{1}{T_1} - \frac{1}{T_2}$$

ΔH can thus be obtained from the slope of a plot of ln K versus $1/T$. When the interaction is exothermic (i.e., driven by a large negative ΔH), the affinity decreases with increasing temperature. The enthalpic quantity ΔH can also be obtained by calorimetric measurement of the heat of reaction (Wiseman et al., 1989; Kelley et al., 1992).

A detailed review of the thermodynamics of antigen–antibody interactions has been published by Mukkur (1984). Examples of the uses of thermodynamic calculations for analyzing immunological interactions are provided by Novotny et al. (1989) and Kelley et al. (1992). Although operational aspects inherent in the various techniques make the absolute values calculated for equilibrium and rate constants somewhat uncertain, comparative binding measurements on antigen or antibody point mutants provide relative affinity data that are very useful for analyzing the thermodynamics of antigen–antibody binding (Kelley and O'Connell, 1993).

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