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Multiple Binding of Antibodies to Antigens: Effect on Radioimmunoassay Binding Curves†

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ABSTRACT: Factors affecting the binding curves measured by radioimmunoassay are examined by comparing theoretical binding curves calculated for ideal populations of homogeneous antibodies and antigens in order to test the conclusions of Berzofsky et al. [Berzofsky, J. A., Curd, J. G., & Schechter, A. N. (1976) *Biochemistry 15*, 2113–2121] about the effect of multiple antigenic determinants on the shapes of such curves. When such ideal binding curves with the same midpoints and limiting values expected for univalent and multivalent antigens are compared, it is clear that multivalent antigens do *not* produce abnormally steep binding curves but rather tend somewhat to produce less steep curves. Under practical experimental circumstances, the binding curves for univalent and multivalent antigens are predicted to be indistinguishable in shape. Some alternative causes of abnormally steep binding curves are suggested.

Antibodies directed against proteins are extremely useful tools in the analysis of protein conformation in solution, especially since such analysis was given a quantitative basis by Sachs et al. (1972). Antibodies recognizing the native conformation of a protein have been used to measure the tendency of protein fragments to adopt that conformation (Sachs et al., 1972; Hurrell et al., 1977; Chavez & Scheraga, 1979), while antibodies against unfolded polypeptides have been used to probe the transient spontaneous unfolding of the native protein (Furie et al., 1975; Hurrell et al., 1977). Antibodies against both native and unfolded conformations have been used to characterize trapped intermediates in protein folding (Creighton et al., 1978; Creighton, 1979).

Although immunochemical analysis is simple in theory, the experimental measurements are made with a complex system. For example, antisera produced against even a simple molecule will generally contain a spectrum of antibody molecules, with varying affinities and in varying proportions. Furthermore, complex molecules such as proteins will elicit such heterogeneous antibody populations against each of several different antigenic sites on the molecule, some of which may overlap, while others are distinct and separate (Crumpton, 1974; Atassi, 1979). Protein antigens may then bind simultaneously two or more antibody molecules against nonoverlapping antigenic sites. Antibody molecules are also multivalent, with two identical binding sites per molecule for the most common IgG class but ten for the IgM class. Indeed, the common formation of an immune precipitate requires the simultaneous binding of at least three antibody molecules to at least some antigen molecules. Consequently, quantitative measurements of the interactions between antibodies and proteins may not be straightforward.

The effect on the radioimmunoassay method of multiple antigenic determinants was examined theoretically and experimentally by Berzofsky et al. (1976) in an attempt to explain an unexpectedly steep binding curve observed experimentally. They concluded that the shape of such a curve "is very sensitive to the multiplicity of determinants" (p 2113), that "as the number of sites n on the antigen molecule increases, the steepness of the slope increases rapidly" (p 2115), and that "classical Scatchard analysis will lead to meaningless numbers" (p 2120) for the affinity between antibodies and a multivalent antigen. This analysis was used recently by Chavez & Scheraga (1979) as one method of estimating the number of antigenic sites on ribonuclease. However, comparison of comparable binding curves calculated for univalent and multivalent antigens shows the primary conclusion of Berzofsky et al. (1976) to be inappropriate. This will be demonstrated here by comparing radioimmunoassay binding curves calculated for antigens with single antigenic sites to those calculated for antigens with up to 10 independent antigenic sites, using the equations of Berzofsky et al. (1976) and the most simple, ideal case where all the populations of antibody molecules have the same concentrations and affinities for their respective antigenic sites. It will be shown by comparing appropriate binding curves that the presence of multiple antigenic sites generally has very little effect on the shape of the curve and that where there is an effect, it is *opposite* to that predicted by Berzofsky et al. (1976).

Radioimmunoassay of a Single Antigenic Site by Homogeneous Antibodies. Binding curves measured by radioimmunoassay express the degree of binding of a small, constant amount of radioactive antigen by a constant amount of an-

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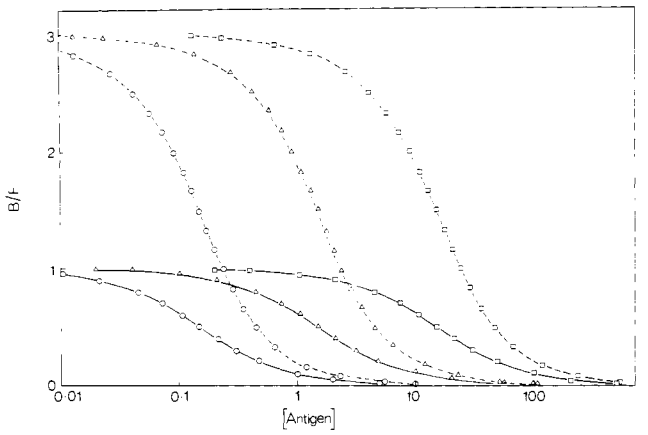

FIGURE 1: Representative ideal binding curves predicted for binding of a univalent antigen by a homogeneous population of antibodies, with dissociation constant K_d and constant concentration A. The ratio of bound (B) to free (F) antigen is plotted as a function of total concentration of antigen on a logarithmic scale. The three solid curves with $(B/F)_{lim} = 1$ were calculated for $A = K_d = 0.1$ (○), $A = K_d = 1$ (△), and $A = K_d = 10$ (□). The three dashed curves with $(B/F)_{lim} = 3$, but the same midpoints as above, were calculated with $A = 0.18$ and $K_d = 0.06$ (○), $A = 1.8$ and $K_d = 0.6$ (△), and $A = 18$ and $K_d = 6$ (□). The symbols give the calculated points. The values of A and K_d are in the same arbitrary concentration units.

tibody as a function of varying concentrations of unlabeled antigen. The labeled and unlabeled antigens compete for the antibody combining sites as a function of their relative concentrations and affinities. The degree of binding of the labeled antigen is measured experimentally by the relative proportions of radioactivity bound to the antibodies and that free in solution. When the unlabeled and labeled antigens are identical, as will be assumed here, the binding of the labeled antigen reflects that of the entire antigen population. The very many theoretical and practical aspects of this procedure have been discussed extensively; references may be found in Feldman et al. (1972), Felber (1974), Rodbard & Frazier (1975), and Orth (1975).

The simplest case involves a single homogeneous population of univalent antibody molecules binding to a single site on a univalent antigen to produce the corresponding complex:

$$\text{antigen} + \text{antibody} \rightleftharpoons \text{complex} \quad (1)$$

The affinity of this interaction may be expressed by the dissociation constant:

$$K_d = (A - C)(L - C)/C \quad (2)$$

where A and L are the *total* molar concentrations of antibody combining sites and antigenic sites on the ligand, respectively, and C is the concentration of the complex.

Radioimmunoassay binding curves are generally expressed as the ratio of bound (B) to free (F) labeled antigen as a function of total antigen concentration:

$$B/F = C/(L - C) = (A - C)/K_d \quad (3)$$

Given constant values of A and K_d, different values of B/F are obtained with varying values of C, which are observed with varying concentrations of total antigen, L. The values of L required for the various values of C may be calculated from eq 2.

Representative theoretical binding curves are illustrated in Figure 1. At very low total antigen concentrations, the value of B/F is at a maximum limiting value, $(B/F)_{lim}$, which should depend upon only the concentration of antibodies and their affinity for the antigen:

$$(B/F)_{lim} = A/K_d \quad (4)$$

Increasing amounts of antibody should increase this limiting plateau. In practice, the precise values of A and K_d are not known, but the antibody preparation is merely diluted to give a convenient value of $(B/F)_{lim}$.

As the total concentration of antigen increases, the antibody combining sites become saturated, and the proportion of antigen bound decreases asymptotically to zero. The concentration of antigen at which the value of B/F decreases to half its limiting value, L_m, is given by

$$L_m = A/2 + K_d \quad (5)$$

The values of $(B/F)_{lim}$ and L_m are sufficient to define unique values of A and K_d, and vice versa, in the ideal situation. Different absolute values of A and K_d, but the same relative values, so that the height of the limiting plateau remains constant, give binding curves of exactly the same shape, when B/F is plotted vs. log L, but shifted horizontally, so that the values of L_m differ. Consequently, there is a unique shape of such binding curves for any given value of $(B/F)_{lim}$. The slope of such a binding curve is taken here as $d(B/F)/d \log L$ at approximately the midpoint L_m. Its value depends only upon this limiting plateau; increased slopes are produced by increased values of $(B/F)_{lim}$, which may be produced merely by increasing the antibody concentration (eq 4).

In reality, radioimmunoassay is not so simple: the active antibodies are usually bivalent, heterogeneous, and of unknown concentration and affinity; large antigens are usually multivalent. The multivalency of antibodies is probably inconsequential for normal radioimmunoassay since it is the distribution of antigen between that in solution and that bound to antibodies which is measured, so long as the multiple binding sites on the antibody molecule are independent; cooperative interaction between antibody binding sites has been considered by Carayon & Carella (1974). Heterogeneity of antibodies can produce complex, multistepped binding curves due to titration of the individual subpopulations. The concentrations and affinities of antibodies are usually not determined, but an amount which gives a convenient value of $(B/F)_{lim}$ is used in the assay. Consequently, the precise value of $(B/F)_{lim}$ observed is not significant.

In contrast, the multivalency of antigens is significant for radioimmunoassay in that it distinguishes between only antigen molecules free in solution, with no antibody molecules bound, and the remainder, with one or more bound antibody molecules, irrespective of the actual number (Berzofsky et al., 1976).

Multiple Binding of Antibody Molecules to a Multivalent Antigen. (1) Effect on Shape of Binding Curves. When an antigen binds more than one antibody molecule independently at nonoverlapping sites, each interaction can be treated as above for the ideal case. Berzofsky et al. (1976) used probability analysis to estimate the proportion of antigen being free, with no antibodies bound; their procedure will be followed here.

The probability of an individual antigenic site being occupied by an antibody molecule is given by

$$b \equiv C/L = [1 + K_d/(A - C)]^{-1} \quad (6)$$

where A, K_d, and C refer to the specific antibodies for the individual antigenic site. The probability of an antigen being free will be the product of the probabilities of each of its n independent antigenic sites being free. For the simplest case, where each antibody against the n antigenic sites is present at the same concentration and has the same affinity, the probability of being bound will be the same for each site (eq

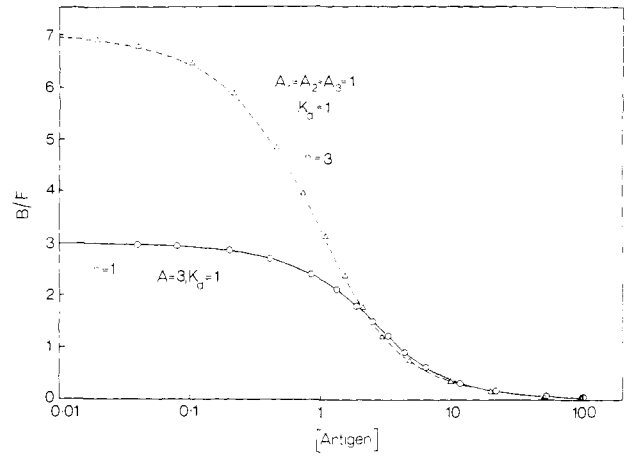

FIGURE 2: Ideal binding curves calculated for binding of the same total concentration of antibodies with the same affinity to a univalent antigen (n = 1) and three individual antibody populations to three independent antigenic sites on the antigen (n = 3). The curve for n = 1 was calculated as in Figure 1 for A = 3 and K_d = 1, while that for n = 3 was with $A_1 = A_2 = A_3 = 1$ and K_d = 1 for each. The values of A and K_d are in the same arbitrary concentration units.

6), and the ratio B/F will be given by eq 10 of Berzofsky et al. (1976):

$$B/F = [1/(1 - b)]^n - 1 \quad (7)$$

A theoretical binding curve for n = 3 is illustrated in Figure 2, where the three antibody populations have the same concentrations of combining sites ($A_1 = A_2 = A_3 = 1$)[1] and the same affinity for their respective antigenic sites (K_d = 1), and compared to the binding curve expected for the same total concentration of antibody (A = 3) directed against a single antigenic determinant with the same affinity (K_d = 1). Multiple binding of antibodies produces a higher limiting value of B/F than would be expected for a univalent antigen, as the probability of having at least one antibody molecule bound is increased. However, the limiting value of B/F expected for univalent or multivalent binding is generally not known a priori because the concentrations and affinities of the antibody combining sites (eq 4) are not known with sufficient accuracy.

That the binding curve for n = 3 has a greater slope than that for n = 1 is of no significance in itself, as this is to be expected for a curve with a higher limiting plateau value. It is not of practical relevance to compare two such curves of different limiting plateaus, as the values to be expected are generally not known. The appropriate question to ask is whether the shapes of the binding curves of multivalent antigens are significantly different from those of the normal curves expected for univalent antigens.

When the above curve for n = 3 is compared to one calculated for n = 1 with the same values of $(B/F)_{lim}$ and the same midpoint (Figure 3), it is clear that the multivalent antigen gives a curve which is somewhat *less* steep. With greater multivalency (n = 10), there is a larger effect, so multivalency of antigens tends to produce *less* steep binding curves than comparable curves expected for univalent antigens.

The difference between the binding curves for multivalent and univalent antigens diminishes with lower limiting values of B/F; the curves for n = 1 and n = 3 are virtually identical when $(B/F)_{lim}$ = 0.7 (Figure 3c). Greater differences are expected with higher values of $(B/F)_{lim}$, but experimental errors are also much greater. Consequently, the differences

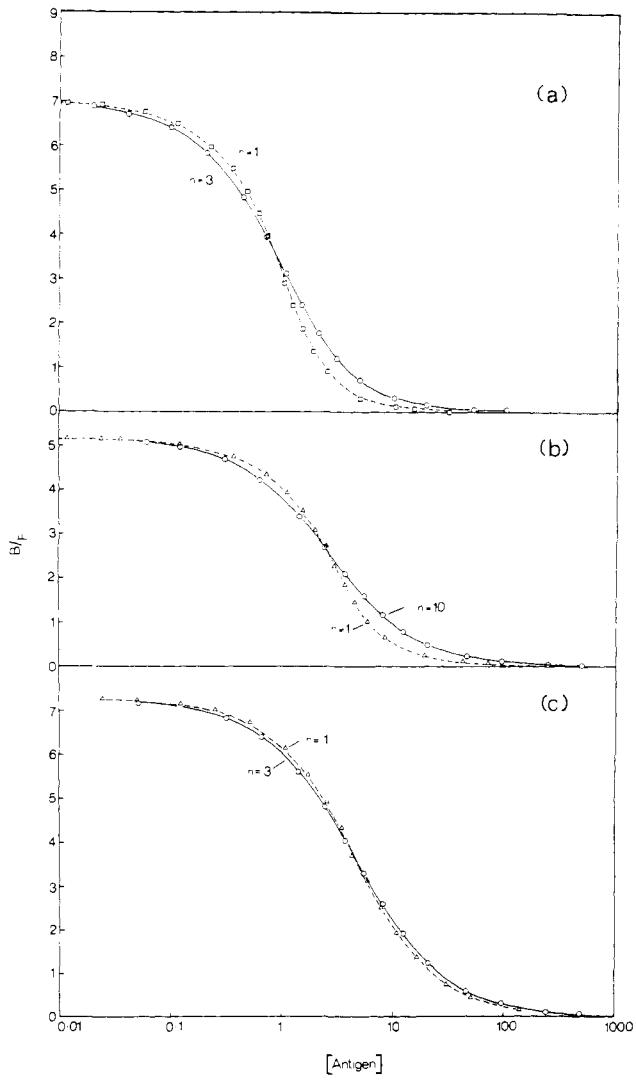

FIGURE 3: Comparisons of binding curves with the same midpoints and plateau values for univalent and multivalent antigens. (a) The curve for n = 3 was calculated with each of the three antibody populations present at a concentration of 1 and with K_d = 1, as in Figure 2, both values in the same arbitrary concentration units. The comparable curve for n = 1 was calculated as in Figure 1 with A = 1.3689 and K_d = 0.1956. (b) The curve for n = 10 was calculated with values of the concentrations and dissociation constants of each antibody population of 1 and 5, respectively, while the comparable curve for a univalent antigen had A = 3.77 and K_d =0.725. (c) The curve for n = 3 was calculated with values of the concentrations and dissociation constants of each of three antibody populations of 1 and 5, respectively, while the comparable curve for n = 1 had A = 2.46 and K_d =3.378. In each case, the symbols indicate the calculated points.

between curves for multivalent and univalent antigens are small compared to the usual experimental error, and it is unlikely that such differences could be detected experimentally.

This demonstration that multivalent antigens tend to give less steep binding curves than univalent antigens contrasts with the conclusion of Berzofsky et al. (1976) that this phenomenon will produce unexpectedly steep slopes. Their erroneous conclusion probably arose because their mathematical analysis did not compare the slopes of comparable curves, with the same limiting value of B/F.[2] It is also clear that the degree of

[1] Values for the molar concentrations of antibody combining sites (A) and for dissociation constants (K_d) are expressed in the same concentration units, which are arbitrary.

[2] It may also be pertinent that Berzofsky et al. (1976) calculated the slopes of binding curves as $d(B/F)/dL$, whereas the binding curves are plotted as B/F vs. log L; the slope of such a curve is given by $d(B/F)/d \log L$.

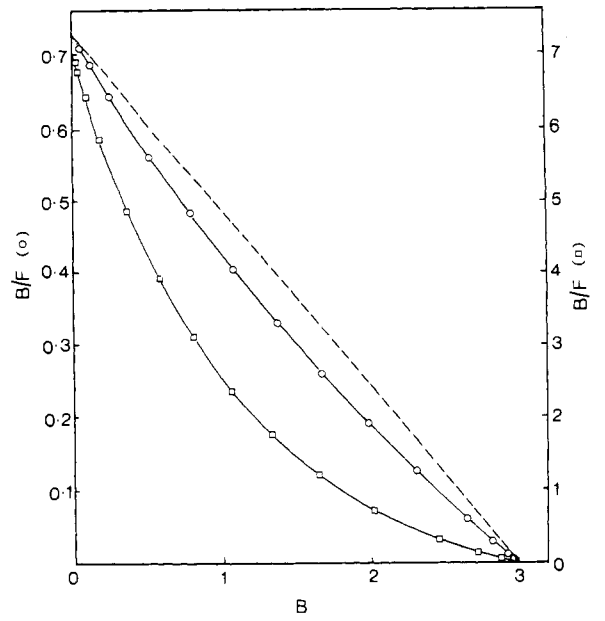

FIGURE 4: Scatchard plots of radioimmunoassay results predicted for independent binding of three antibody molecules of equal affinities and concentrations to three sites on an antigen. B is the concentration of antigen bound to antibodies, while F is that of free antigen. The curve O was calculated with $K_d = 5$ and $A_1 = A_2 = A_3 = 1$, as in Figure 3c; the curve □ differed only in having $K_d = 1$. The curves O and □ refer to the B/F scales on the *left* and *right*, respectively. The dashed line is the linear curve expected for ideal binding of univalent antigen and antibody.

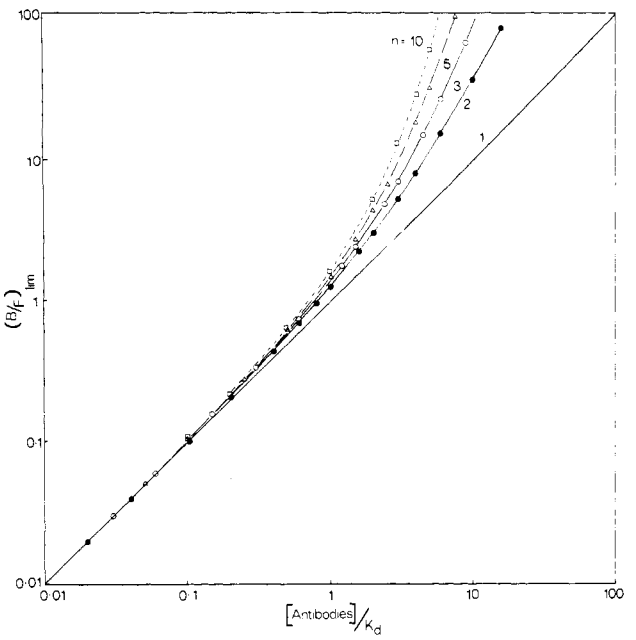

FIGURE 5: Predicted dependence of the limiting value of B/F on antibody concentration for independent binding of antibodies to n antigenic sites. The n antibody populations were taken to have the same affinity for the antigen and to be present at the same concentrations. The total concentration of antibody is plotted as the dimensionless ratio to the dissociation constant, K_d.

multivalency of an antigen cannot be determined from the shape of such a binding curve [cf. Chavez & Scheraga (1979)] without other information.

(2) *Other Effects.* Multiple binding of antibody molecules tends to produce nonlinear Scatchard plots of radioimmunoassay data and to give erroneous estimates of the antibody affinities, as predicted by Berzofsky et al. (1976). Estimation of antibody concentration and affinity from binding curves using eq 4 and 5 will be erroneous for multivalent antigens. However, such effects are relatively small with low limiting values of B/F; two Scatchard plots for $n = 3$ are illustrated in Figure 4, for high and low values of $(B/F)_{lim}$ (parts a and c of Figure 3). The nonlinear curves would normally be interpreted as heterogeneity of binding affinities; in the case of $(B/F)_{lim} = 0.73$, the values of K_d would be estimated to vary from approximately 3 to 6, whereas all the antibodies present were given $K_d = 5$. The range of apparent affinities is greater with $(B/F)_{lim} = 7$, from about 0.2 to 2, while the antibodies were given $K_d = 1.0$ Scatchard analysis of such data will give misleading values of K_d, although this may be minimized by using a low antibody concentration, so that $(B/F)_{lim}$ is no greater than ~ 1.

The most characteristic effect of multiple binding of antibody molecules appears to be the nonlinear dependence of the limiting value of B/F upon the concentration of antibody. This may be calculated from the maximum probability of binding an antibody at each site, b_{max}:

$$b_{max} = (1 + K_d/A)^{-1} \quad (8)$$

When the n antibody populations have the same affinity and concentration, the limiting value of B/F as a function of n and b_{max} is obtained from eq 7:

$$(B/F)_{lim} = [1/(1 - b_{max})]^n - 1 \quad (9)$$

A plot of this value as a function of total antibody concentration for $n = 1$, 2, 3 5, and 10 is shown in Figure 5. The curves diverge and become increasingly nonlinear with increasing n at high antibody concentrations, i.e., high values of B/F. In principle, it would be possible to determine the value of N experimentally from the shape of such a curve, but the differences seem too small, especially since experimental errors become increasingly important at high values of B/F.

There appears to be no feasible way of determining the value of n from radioimmunoassay data alone.

(3) *Minimizing the Effects of Multivalency.* The anomalous effects on radioimmunoassay data of multiple binding of antibody molecules become minimal at low values of B/F, particularly when the antigen concentration is sufficiently great so that the antibody combining sites are limiting and saturated. Under these conditions, each antigen molecule is likely to have at most a single antibody molecule bound to it, and multiple binding will be negligible. Labeled and unlabeled antigen molecules then compete for the limiting antibody combining sites as if they were univalent ligands.

It is recommended that radioimmunoassay measurements of phenomena such as protein conformational equilibria be performed where the concentration of labeled antigen alone is sufficiently high to saturate all antibody combining sites. Besides minimizing multiple binding of antibody molecules, these conditions also minimize the effects of antibody heterogeneity, because the actual values of the affinity constants are irrelevant, provided only that they are great enough to ensure saturation of all the antibody combining sites. This procedure, which has been demonstrated experimentally (Creighton et al., 1978), need limit only slightly the sensitivity of the radioimmunoassay, as it is always limited by the intrinsic affinities of the antibodies. It is also considerably simpler than the alternative of preparing monospecific antibodies, as suggested by Berzofsky et al. (1976).

Other Explanations of Steep Binding Curves. The steep binding curve observed experimentally by Berzofsky et al. (1976) remains unexplained, so additional factors which might affect the shapes of such curves have been considered. Heterogeneity of antibodies, if expressed in varying affinities,

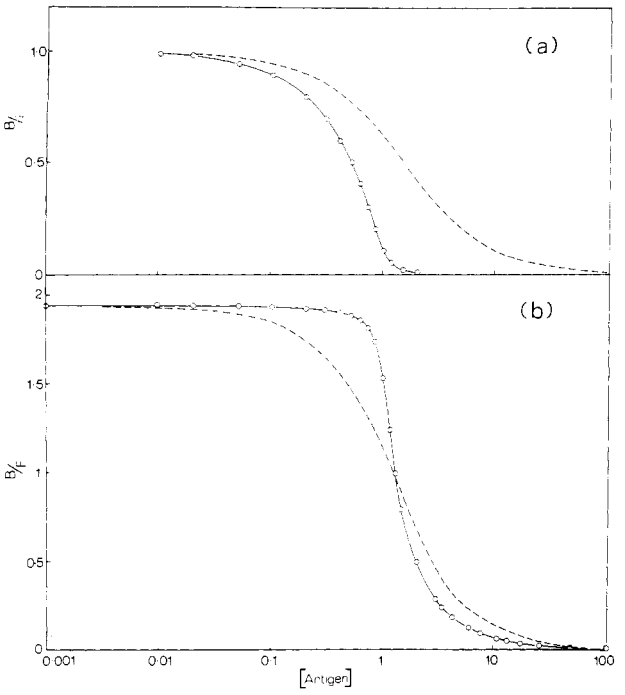

FIGURE 6: Examples of predicted binding curves with steep slopes due to (a) the unlabeled antigen having higher affinity for antibody and (b) incomplete binding of antigen. In (a), the dashed curve was calculated for normal binding of a univalent antigen to homogeneous antibody present at a concentration of 1 and with a dissociation constant of 1, both in the same arbitrary concentration units. The other curve was calculated for the same antibodies and labeled antigen, which was present at a constant, much lower concentration, but where the unlabeled antigen, added to vary the total concentration of antigen, bound to the antibodies with 100-fold greater affinity (dissociation constant of 0.01). Higher relative affinities have no greater effect on the shape of the curve. In (b), one-third of the labeled antigen could not be bound by the antibodies. The remainder of the antigen was homogeneous and univalent, the antibody population was homogeneous and present at a concentration of 1 (arbitrary units), and the 1:1 complex had a dissociation constant of 10^{-2} (same arbitrary units). The normal limiting value of B/F would be 100, but the apparent value is lowered to 2 by the radioactive antigen contaminant. The dashed curve is the normal curve for univalent antigen and antibody with the same limiting value of B/F and the same midpoint.

will tend to decrease the steepness of such curves. Cooperative binding of antibodies to multiple sites on an antigen is especially likely to occur with antibodies directed against proteins, in view of the general cooperative nature of protein conformational equilibria (Privalov & Khechinashvili, 1974). However, it would seem unlikely to affect radioimmunoassay measurements, which measure only the binding of the first antibody molecule. This was confirmed by calculating ideal binding curves for the totally cooperative, all-or-none binding of n different antibody molecules, each of which was taken to be present at the same concentration. Curves calculated with all-or-none binding of 2–10 antibody molecules were indistinguishable in shape from those calculated with independent binding, as in Figure 3. Cooperativity between antibody combining sites may produce steeper binding curves, but this must be accompanied by the absence of a limiting plateau value of B/F, which will decrease with lower concentrations of antigen (Carayon & Carella, 1974).

Two factors which should produce unexpectedly steep binding curves are (a) greater affinity of the antibodies for the unlabeled antigen and (b) incomplete binding of the radioactive antigen.

(*1*) *Greater Affinity for Unlabeled Antigen.* Berzofsky et al. (1976) reported a personal communication from D. Rodbard that a difference in affinity of antibody for labeled and unlabeled antigen may produce a steepening of slope. This has been confirmed when the unlabeled antigen has higher affinity (Figure 6a), but lower affinity has the opposite effect. When the radioactive antigen has been labeled by modifying covalently the unlabeled antigen, as is usually the case, a decrease in affinity is likely, so somewhat steeper binding curves might be expected in many cases. However, the theoretical curve becomes relatively steeper at low values of B/F, whereas the curve considered by Berzofsky et al. (1976) was most steep at high values.

(*2*) *Incomplete Binding of Radioactive Antigens.* The experimental procedure used may be incapable of measuring fully the extent of binding, due to insufficient time being allowed to reach equilibrium, incompleteness of the precipitation procedure genereally used to separate bound from unbound antigen, or dissociation of the antigen after precipitation. Also, the labeled antigen might be impure, with a fraction of the radioactivity incapable of being bound by the antibody preparation. These factors will place a limit on the maximum value of B/F which may be measured, which will be reached with high antibody concentrations and low antigen concentrations. As the total concentration of antigen is increased to saturate the antibody combining sites, a very abrupt titration curve will result. An example for a univalent antigen is presented in Figure 6b, where one-third of the labeled antigen was imagined to be a contaminant. This gives a limiting value for B/F of 2, whereas the concentration of antibody was such that a much higher limiting value (100) would have been otherwise expected. The shape of this theoretical curve is very similar to the steep experimental curve considered by Berzofsky et al. (1976).

The factors considered here are just a few of the many likely to make experimental radioimmunoassay curves differ from those predicted theoretically for ideal situations. It is very likely that other such circumstances could be envisaged which will produce unexpectedly steep radioimmunoassay binding curves.

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