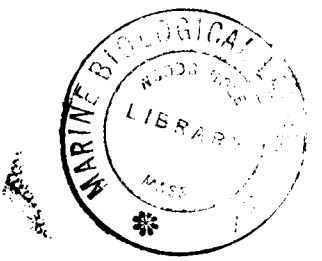
COMPUTER SIMULATION OF RADIAL IMMUNODIFFUSION

II. SELECTION OF AN ALGORITHM FOR THE ANTIBODY-ANTIGEN REACTION IN GELS



RODES TRAUTMAN

From the Plum Island Animal Disease Laboratory, Veterinary Sciences Research Division, Agricultural Research Service, United States Department of Agriculture, Greenport, New York 11944

ABSTRACT An algorithm needed for computer simulation of immunodiffusion has been deduced from existing theories of the in vitro reaction between antibody and antigen. The "Goldberg most probable polymer distribution" theory provides a formula that gives the amount of free antibody, free antigen, and diffusible complexes from extreme antibody excess through extreme antigen excess for any valences of antibody and antigen. As is shown here, that formula can be used even for those reactions producing complexes, cyclical or otherwise, that may precipitate as well as for those reactions involving heterogeneity of binding avidities. It is necessary, however, to specify an extent of reaction parameter. Five limiting expressions for this parameter are proposed as options for the basic algorithm. These are identified as: (*a*) the "Heidelberger-Kendall complete reaction" option, (*b*) the "Singer-Campbell constant avidity" option, (*c*) the "Hudson extensive antibody heterogeneity" option, (*d*) a new "extensive antigen heterogeneity" option, and (*e*) the "Goldberg critical extent of reaction" option. Literature data showing need for the various options are presented.

INTRODUCTION

For computer simulation, general qualitative ideas must be expressed mathematically (Martin, 1968). When this is done, the sequence of computational steps that gives the solution to all problems of a specified type is called an algorithm. Two fundamental algorithms are required for simulation of immunodiffusion: one for the chemical reaction between antibody and antigen molecules with possible formation of precipitates as well as soluble complexes, and a second one for the diffusion of free antibody and antigen molecules and their soluble complexes. The results of reviewing the literature in order to extract a suitable algorithm for the chemical reaction are given in this paper. The diffusion algorithm is given in part I (Trautman, 1972), and the results of combining the algorithms for single-well

radial immunodiffusion (Mancini et al., 1965; Vaerman et al., 1969; Trautman et al., 1971) will be reported in part III.

APPROACH

A fundamental concept involved in statistical mechanical models of the highly specific antibody-antigen reaction in vitro is that antibodies and antigens can be taken as chemicals of definite valence, no matter in what proportions the antigen and antibody are mixed. Numerous complications in this model can be visualized: (*a*) an antigen, instead of having identical combining sites, may have several different antigenic determinants so that the antiserum produced contains a mixed population of antibodies; (*b*) antibody sites, specific for the same determinant, may vary in reactivity; (*c*) the various sites on the same antigen molecule may overlap; (*d*) one antibody may attach by several sites to the same antigen molecule; and (*e*) the same bonds may not be formed in replicate final mixtures, especially if prepared stepwise. Nevertheless, Aladjem and Palmiter (1965) state that there are only two possibilities for each site: it is either filled, i.e. reacted, or it is empty. They further observed that these two states exist independently both of how strong (avid) the bond might be when formed and of the presence of heterogeneity of binding avidities.

The approach taken here is (*a*) to consider the number of antigen molecules times their valence as the maximum number of bonds that could ever be formed; (*b*) to count the number of bonds that have actually formed whether or not they are between the same physical sites involved in establishing the valence; (*c*) to express the extent of reaction as the ratio of the number in *b* to that in *a*; (*d*) to consider the complications listed above as influencing the extent of reaction and not the valence; (*e*) to divide the complications into extreme categories, represented by different options for computing the extent of reaction; and (*f*) to assume that the reaction achieves the extent specified in the option selected.

Palmiter and Aladjem (1963) pointed out that in the analysis of antibody-antigen reactions one can consider either the soluble complexes or those that precipitate. In immunodiffusion, because complexes larger than a specified size will be trapped in the gel, it is unnecessary to establish conditions for actual precipitation. The goal, then, is to find a suitable algorithm for computing the amounts of small complexes that permits any valence of antigen, any valence of antibody, heterogeneity of reagents, as well as cyclical complex formation. Fortunately, the ideas expressed mathematically by Heidelberger and Kendall (1935), Karush (1956), Goldberg (1952), Singer and Campbell (1953), Talmage and Cann (1961), Amano et al. (1962), Palmiter and Aladjem (1963), Aladjem and Palmiter (1965), and Hudson (1968) can be synthesized into an algorithm with several options. The proper names applied to the options are not intended to imply that the persons named consider the option either theirs or universally applicable. Comments on omissions, lack of

proper credit, and, especially, data that indicate need for additional options are welcome.

GOLDBERG MOST PROBABLE POLYMER DISTRIBUTION THEORY

Statistical Mechanical Basis

Goldberg (1952, 1953) seems to have been the first to adapt the statistical mechanical theories of polymer chemistry to antibody-antigen reactions. Palmiter and Aladjem (1963), Amano et al. (1962), and Aladjem and Palmiter (1965) have made notable elaborations. The papers are indeed quite complex and require a high degree of mathematical sophistication on the part of the reader. The term "polymer" is used here, advisedly, not only to indicate the origin of the theory but to emphasize the model used for the statistical mechanics. This model is a "copolymerization" of two distinct types of "monomeric" units of definite but different valence: antibody (Ab) of valence g and antigen (Ag) of valence f, with the restrictions that bonds can be formed only between Ab and Ag valence sites (abbreviated to "sites") and that valences must be integers.

It is important to note the following. (*a*) The statistical mechanical relationships were all derived on the basis of the numbers of molecules, not of concentrations. (*b*) The statistical mechanical formulas are independent of heterogeneity. (*c*) The same statistical mechanical model was used by Palmiter and Aladjem (1963) and Aladjem and Palmiter (1965). (*d*) A second level of models is required for the evaluation of the parameters that were introduced at the statistical mechanical level. (*e*) Some of the second-level models use concepts of thermodynamics that depend both on concentrations and selection of constituents, and some involve precipitation. (*f*) Variations in the second-level models constitute the controversial aspects of the several authors' contributions. (*g*) Goldberg considered any valence of antibody; the other works are restricted to bivalency.

Polymers are called physically distinct if they are not cross-linked through reacted sites, even though they may be branched and entangled. Such polymers and the unreacted monomers are sorted, hypothetically, into various categories, with the set of counts constituting a description of the polymer distribution. The Goldberg (1953) classification scheme defines m_{ik} ($i \geq 0$, $k \geq 0$) as the number of polymers with i Ab and k Ag units. The Amano et al. (1962) scheme also considers the number of polymers with k Ag units but as further classified by the number of Ab that are bound specifically at each Ag site. Palmiter and Aladjem (1963) and Aladjem and Palmiter (1965) classify polymers according to the numbers of reacted sites on both the Ab and Ag constituents. Because of these various schemes it is necessary to consider the extent of reaction in greater detail.

Let A, X be the numbers of monomers in the system of Ab and Ag types, respectively, before the reaction; r, the ratio of total Ag sites to Ab sites, $r = fX/(gA)$; p,

the extent of reaction, i.e. fraction of initially available Ag sites that are filled (reacted); M, the total number of physically distinct polymers, including unreacted monomers; $\{M\}$, the set of numbers according to a specified scheme describing the polymer distribution; and $\Omega(\{M\})$, the number of physically distinct ways to form a polymer distribution with the set of numbers $\{M\}$.

A measure of the relative probability that a polymer distribution $\{M\}$ can exist is taken as $\Omega(\{M\})$. The most probable polymer distribution of all the possible $\{M\}$ in any specified scheme for a specified total M is defined as the one that has the greatest number of physically distinct ways of combining the monomers of the system. The criterion involved is written in these two ways:

$$\begin{aligned} d\Omega(\{M\}) &= 0, \\ (1/\Omega)d\Omega &= d\ln\Omega = 0. \end{aligned} \tag{1}$$

What is not made clear by any of the authors is whether nature selects the most probable distribution on the basis of (*a*) a constant extent of reaction p, or (*b*) a constant number of physically distinct polymers M. All authors do show, however, that if only linear and branched chain polymers are permitted, i.e. cyclical polymers are forbidden, a constant M implies a constant p, and vice versa.

The evaluation of the differential in Eq. 1 requires partial derivatives, which are subject to certain constraints. No matter what the classification scheme used, all authors required M and the total amounts of Ab and Ag to be constant. No further conservation of mass constraints were needed by Goldberg (1952, 1953), who assumed homogeneity for both reagents. Amano et al. (1962) required a constant total number of each of the different Ab kinds, represented by their specificity to each antigen site. Palmiter and Aladjem (1963) considered homogeneous antibody reacting with possibly different avidities to the different antigen sites, and Aladjem and Palmiter (1965) considered heterogeneous antibody reacting with possibly different avidities not only to different antigen sites but also to the same site. Heterogeneity of either or both reagents merely changed the constraints and not the statistical mechanical concepts. The important point in the search for an algorithm is that the most probable polymer distribution for any overall extent of reaction is simultaneously the most probable distribution for individual extents of reaction for each of the identified sites, even with heterogeneity present. The problem is to apply this principle to give an expression for $\{M\}$ for any particular classification scheme.

Solution of Statistical Mechanical Problem

Consider now the special Goldberg case. The probability argument suggested by Talmage and Cann (1961) for bivalent Ab can be generalized and used to give a partial solution, without using the mathematics of Eq. 1.

The probability that an Ag monomer will have all of its f sites unreacted, i.e. be

free, is the product of the probability for each site, or $(1 - p)^f$. Hence, $m_{01}/X = (1 - p)^f$. Similarly, the fraction of unreacted Ab sites is $(1 - rp)$ whatever the value of p, since rp is the "extent" of Ab reaction. The probability of having all g sites on an Ab monomer unreacted will be $(1 - rp)^g$ and $m_{10}/A = (1 - rp)^g$. There is a product of three probabilities involved in the occurrence of the AbAg complex: the probability that any site on Ab has reacted with Ag, $g(rp)$; the probability that its other $g - 1$ sites are unreacted, $(1 - rp)^{g-1}$; and the probability that the other $f - 1$ Ag sites are also unreacted, $(1 - p)^{f-1}$. Thus, $m_{11}/A = (grp)(1 - p)^{f-1}(1 - rp)^{g-1}$. The importance of these formulas is that they do not appear to require any assumptions about the reaction, other than that it is describable by an overall value of p.

In order to achieve the complete solution in terms of a general expression for the amount of any complex, all the authors had to restrict the reaction to linear and branched chain polymers in order to solve Eq. 1. Goldberg's formula for the amount, m_{ik}, of any linear or branched chain compound that has i molecules of antibody and k molecules of antigen, in an algebraically equivalent form in order to emphasize symmetry, is

$$m_{ik} = \beta_{ik}(gA/p)p^i(rp)^k(1 - p)^{fk-k-i+1}(1 - rp)^{gi-i-k+1}, \tag{2}$$

where

$$r = fX/(gA),$$

$$\beta_{ik} = \frac{(gi - i)!(fk - k)!}{(gi - i - k + 1)!(fk - k - i + 1)!i!k!}. \tag{3}$$

Binding Variables

Consider the total amount of antibody bound y, whether as soluble or as precipitated complexes. This will be the total antibody less that which is free. From the above expression evaluated for m_{10},

$$y = A - m_{10} = A[1 - (1 - rp)^g]. \tag{4}$$

Note that both m_{10} and y are independent of antigen valence f. Similarly, the amount of free antigen m_{01} and the amount bound z are both independent of antibody valence g, since

$$z = X - m_{01} = X[1 - (1 - p)^f]. \tag{5}$$

The ratio of antibody to antigen molecules bound in all complexes approaches f/g at "equivalence" ($r = 1$) only if $p = 1$ there, as can be seen from

$$\frac{y}{z} = \left(\frac{f}{gr}\right)\left[\frac{1 - (1 - rp)^g}{1 - (1 - p)^f}\right]. \tag{6}$$

Average binding can be expressed as the number of bound molecules of one reagent divided by the total amount of the opposite reagent. The two forms required are denoted as $\bar{\nu}_{Ab}$ and $\bar{\nu}_{Ag}$ and are given by

$$\bar{\nu}_{Ab} \equiv (A - m_{10})/X = [f/(gr)][1 - (1 - rp)^g],$$
$$\bar{\nu}_{Ag} \equiv (X - m_{01})/A = (gr/f)[1 - (1 - p)^f]. \quad (7)$$

At small values of p, $(1 - p)^f \approx 1 - fp$, and similarly at small values of rp, $(1 - rp)^g \approx 1 - grp$. With these relations, it can be shown that the limiting values for y/z are f for extreme antibody excess ($rp \to 0$) and $1/g$ for extreme antigen excess ($p \to 0$). The corresponding limits for $\bar{\nu}_{Ab}$ and $\bar{\nu}_{Ag}$ are fp and grp, respectively.

Basic Algorithm

No inherent distinction was made between antibody and antigen molecules as chemicals in deriving Eq. 2, except that neither could react with itself. Since formulas 2, 4, and 5 apply in antigen excess as well as antibody excess for any valences, they have been selected as the basis for the antibody-antigen reaction algorithm to be used in the simulation of radial immunodiffusion. For that application, f, g, A, and X are known, so the problem becomes one of selecting the extent of reaction parameter p. This is where the second level of models must be clearly distinguished from the first-level statistical mechanical models. These second-level models will be presented as options in the algorithm.

HEIDELBERGER-KENDALL COMPLETE REACTION OPTION

Empirical Basis

Of the three expressions given by Heidelberger and Kendall (1935) for the antibody excess side of equivalence, the parabolic formula has been widely used as an empirical description rather than as a theoretically sound mass action model. It gives, for the precipitate, the amount of antigen as X and the amount of antibody as $2RX - R^2X^2/A$. Here R is the molar ratio of antibody to antigen in the precipitate at equivalence where $X = A/R$. The extent of reaction involved in this formulation is not immediately apparent, and so will be deduced.

Write Eq. 4 for bivalent antibody as

$$(y)_{g=2} = 2Arp - Ar^2p^2 = fpX - [f^2p^2/(4A)]X^2, \quad (8)$$

where r has been replaced on the right-hand side by its definition, $fX/(gA)$. The Heidelberger-Kendall parabolic expression will result if (*a*) $p = 1$, (*b*) $g = 2$, (*c*) $R = f/2$, and (*d*) all complexes precipitate. Thus, the Heidelberger-Kendall para-

bolic formula implies a complete reaction of all antigen sites ($p = 1$) everywhere on the antibody excess side of equivalence and it applies to bivalent Ab only.

Generalization

The Heidelberger-Kendall complete reaction option will be taken to represent those antibody-antigen reactions involving any valence that are complete for the reagent in shorter supply. This means for $r \leq 1$, $p = 1$, and for $r \geq 1$, $rp = 1$, or compactly

$$p = \text{minimum } (1, 1/r). \tag{9}$$

Using this formula for p, the amount of each reagent bound can be computed from Eq. 4 and 5, and the amount of any particular complex from Eq. 2.

It might appear that for $p = 0$ or 1, Eq. 2 would always yield zero on the right-hand side. However, the exponents also become zero for certain values of i and k, with the corresponding indeterminant factor becoming unity in the limit. Fig. 1 shows the relative amounts of the various complexes that exist for pentavalent Ag and bivalent Ab undergoing a complete reaction.

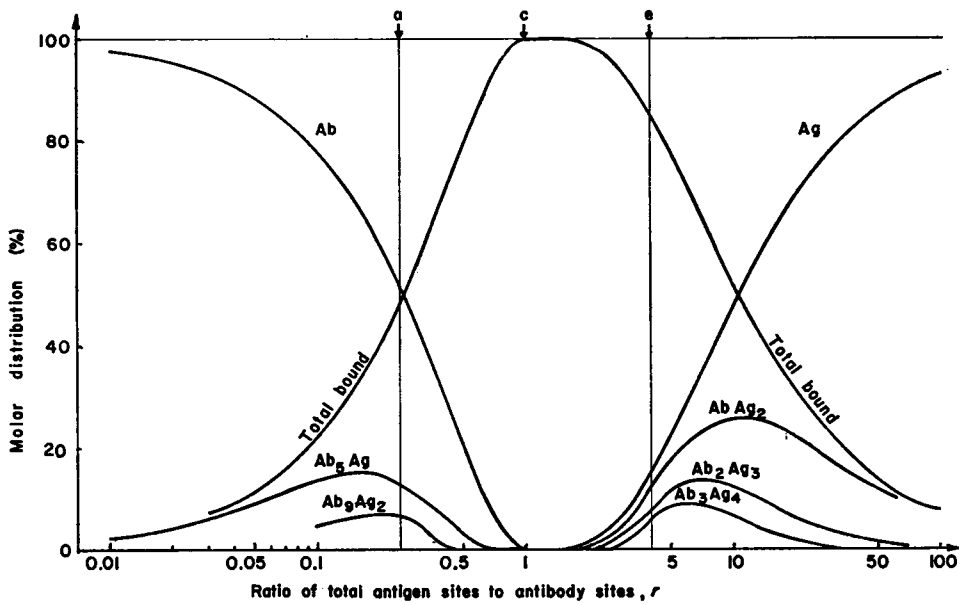


FIGURE 1 Distribution of small complexes for the Heidelberger-Kendall complete reaction option for a pentavalent antigen-bivalent antibody system. The ordinate refers to the number of moles of antibody plus antigen bound in the complex divided by the total number of moles present before reaction, $(i + k)m_{ik}/(A + X)$. Special values along the abscissa are: *a*, Goldberg antibody inhibition of aggregation limit, $r = 1/[(f - 1)(g - 1)]$; *c*, equivalence of numbers of antigen and antibody sites, $r = 1$; and *e*, Goldberg antigen inhibition of aggregation limit, $r = (f - 1)(g - 1)$.

Verification

The successful use of the parabolic formula to fit quantitative precipitin data (Kabat and Mayer, 1961) shows that bivalent antibody systems do exist for which a complete reaction on the antibody excess side is an adequate description. Lindqvist and Bauer (1966) gave precipitin data showing consistency with the generalization for both sides of equivalence and for pentavalent antibody. Their data on the rabbit IgM-bovine serum albumin (BSA) immune system are replotted in Fig. 2. From the graph, it is evident that (*a*) the bound antibody is not resolubilized on the antigen excess side of equivalence; (*b*) the molar ratio in the precipitate extrapolates, as expected, to $f = 2$ in antibody excess and to $1/g = \frac{1}{5}$ in antigen excess; (*c*) this molar ratio has the theoretical ratio $f/g = \frac{2}{5}$ at equivalence; and (*d*) its curve is not straight, since $g = 5$ in Eq. 6.

More detailed applicability of Eq. 2 can be shown by its internal consistency. Thus, determine the extent of reaction from the observed amount of free antigen and the equation for m_{01}; use this to compute the amounts of higher complexes actually measured. This test was suggested by Singer and Campbell (1953), but

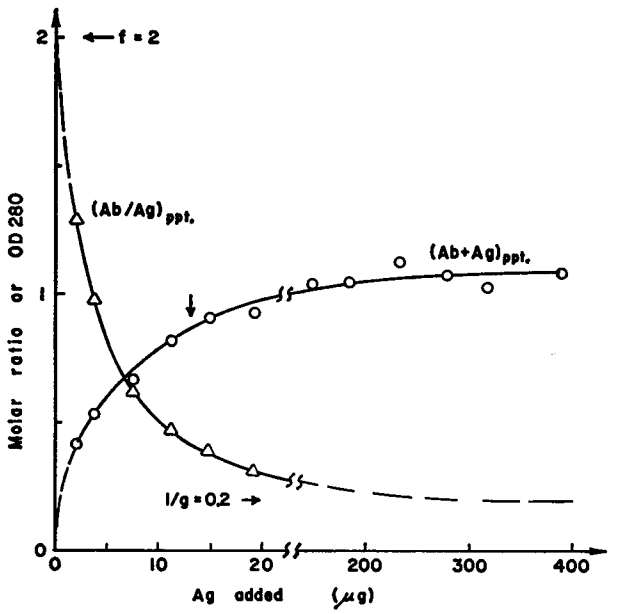


FIGURE 2 Verification of the Heidelberger-Kendall complete reaction option. Quantitative precipitin analysis of BSA-rabbit IgM system that shows a complete precipitating reaction on both sides of equivalence (Lindqvist and Bauer, 1966). Data for authors' Fig. 4 obtained by personal communication; $(Ab + Ag)_{ppt.}$ is total precipitate optical density; $(Ab/Ag)_{ppt.}$ is molar ratio in precipitate assuming all Ag precipitated (cited molecular weights are Ag 69,000 and Ab 900,000); arrow indicates equivalence; extrapolations were not given by authors.

the most convincing data, for antigen excess, have been given by Williams and Donermeyer (1962) (Table I). These data show (*a*) that the absence of any free antibody in the ultracentrifuge patterns means that the antibody reaction was complete; (*b*) this is verified since the computed value of *rp* from the measured amount of free antigen is not significantly different from unity (column 2, Table I); (*c*) the observed percentage distribution of complexes agrees with prediction. Note that the authors extrapolated, to infinite dilution, the data from the series of ultracentrifugal experiments with no evidence presented that the extent of reaction depended on volume at a given value of *r*.

TABLE I

CONSISTENCY OF THE GOLDBERG FORMULATION FOR BSA-CHICKEN IgG SYSTEM IN EXTREME ANTIGEN EXCESS AND AT pH 8.6 WHERE THE EXTENT OF ANTIBODY REACTION IS COMPLETE (WILLIAMS AND DONERMEYER, 1962)

*r**	Extent of antibody reaction, *rp*‡	Percentage distribution of complex§	
		Predicted‖	Observed
25.8	0.95 ±0.08	23.5	31.7
30.5	0.96 ±0.08	22.1	29.3
39.3	1.08 ±0.10	22.4	25.6
39.4	0.92 ±0.10	19.6	24.0
44.4	1.00 ±0.10	18.6	22.7
73.9	1.02 ±0.15	13.7	15.0

* Entire table recomputed from given percentage distribution by ultracentrifugation; *r* is ratio of total antigen sites to antibody sites using $f = 6$, $g = 2$, and molecular weights cited by authors (Ag, 67,000; Ab, 155,000). Globulin precipitated at pH 7.5 contained 40% nonspecific macroglobulin. All solutions were centrifuged at several dilutions and relative areas of schlieren peaks were extrapolated to infinite dilution. The only concentration cited was 18.65 mg/ml for the undiluted run on the second sample.

‡ Computed from the extent of reaction determined by free antigen. The error given represents the change in *rp* for an assumed one percentage point change in the given percentage of free Ag. A complete reaction corresponds to $rp = 1$ with no free antibody. None was detected in any of the samples.

§ The authors did not make this comparison between observed and predicted, but showed instead that the Goldberg theory for $rp = 1$ predicts the correct molar ratio of bound antibody to antigen.

‖ Computed from the extent of reaction assuming complex is $AbAg_2$ and, in addition, AbAg only if $rp < 1$.

SINGER-CAMPBELL CONSTANT AVIDITY OPTION

Thermodynamic Model

Singer and Campbell (1953) were the first to point out that an intrinsic equilibrium "constant" K was implicit in the Goldberg formulation of Eq. 2. Their introduction of thermodynamics converts the search for formulas for the extent of reaction into a search for expressions for K. The line of reasoning involves the following steps.

First, replace each m_{ik} of Eq. 2 by the corresponding molar concentration c_{ik} by dividing by the volume, a reasonable procedure only for nonprecipitated complexes. For example,

$$\begin{aligned} c_{Ab} &\equiv c_{10} = c^0_{Ab}(1 - rp)^g, \\ c_{Ag} &\equiv c_{01} = c^0_{Ag}(1 - p)^f, \\ c_{11} &\equiv gc^0_{Ab}(rp)(1 - p)^{f-1}(1 - rp)^{g-1} \\ c_{12} &\equiv [g(g - 1)/2]c^0_{Ab}(rp)^2(1 - p)^{2f-2}(1 - rp)^{g-2}, \end{aligned} \tag{10}$$

where $r = fc^0_{Ag}/(gc^0_{Ab})$, in terms of initial concentrations rather than amounts.

Secondly, write a selected set of chemical reactions for antigen excess involving an f-valent antigen and a g-valent antibody:

$$\begin{aligned} &Ab + Ag \rightleftarrows AbAg && K_{11} \equiv c_{11}/(c_{10}c_{01}) \\ &AbAg + Ag \rightleftarrows AbAg_2 && K_{12} \equiv c_{12}/(c_{11}c_{01}) \\ &\quad\vdots && \quad\vdots \\ &AbAg_{g-1} + Ag \rightleftarrows AbAg_g && K_{1g} \end{aligned} \tag{11}$$

The equilibrium constant for each reaction is defined by the corresponding equation on the right where, for simplicity, molar concentrations rather than thermodynamic activities are used.

Thirdly, make the analysis tractable by redefining the constituents in terms of sites rather than molecules and write one overall reaction as

$$\text{Ab-site} + \text{Ag-site} \rightleftarrows \text{AbAg-bond} \qquad K \equiv [\text{AbAg-bond}]/\{[\text{Ab-site}][\text{Ag-site}]\}, \tag{12}$$

where K is called the intrinsic equilibrium constant. This formulation means that for a constant K the reaction in terms of sites is independent of how the sites are actually distributed among the molecules present. Thus, if the first two of the reactions of Eq. 11 are expressed in terms of sites

$$\begin{aligned} K &= \frac{c_{11}}{(gc_{10})(fc_{01})} = \frac{K_{11}}{fg}, \\ K &= \frac{2c_{12}}{[(g - 1)c_{11}](fc_{01})} = \frac{2K_{12}}{f(g - 1)}. \end{aligned} \tag{13}$$

Fourthly, substitute Eq. 10 into the first of the Eqs. 13. This provides the fundamental relationship between the extent of reaction parameter and the intrinsic equilibrium constant (Singer and Campbell, 1953; Talmage and Cann, 1961; Hudson, 1968):

$$Q \equiv gc^0_{Ab}K = p/[(1 - p)(1 - rp)] = fc^0_{Ag}K/r. \tag{14}$$

Eq. 14 can be solved explicitly for p, as a general quadratic, without specifying the value of Q at this point (Talmage and Cann, 1961)

$$p = \{1 + Q + rQ - [(1 + Q + rQ)^2 - 4rQ^2]^{1/2}\}/(2rQ), \tag{15}$$

where the positive root is omitted because it gives values of p and rp greater than unity, as can be seen by taking the limit of p as $r \to \infty$, for example.

It should be noted that (*a*) if p or rp is unity (a complete reaction), K is infinitely large in Eq. 14 and cannot be determined experimentally, and (*b*) if p is independent of dilution, Eq. 14 shows that K cannot be constant, and conversely.

In a univalent antigen and g-valent antibody system, the only chemical reactions on both sides of equivalence are those of Eq. 11. The reaction in terms of sites can still be written as Eq. 12. Conservation expressions when $f = 1$ are

$$\begin{aligned}
[\text{Ab-site}]_{\text{free}} &= gc_{Ab}, \\
[\text{Ag-site}]_{\text{free}} &= c_{Ag}, \\
[\text{Ab-site}]_{\text{filled}} &= [\text{AbAg-bond}] = b_{Ag}, \\
[\text{Ab-site}]_{\text{total}} &= gc^0_{Ab} = gc_{Ab} + b_{Ag}.
\end{aligned} \tag{16}$$

Eliminate c_{Ab} (free antibody) from Eqs. 12 and 16 and solve for b_{Ag} (bound antigen) to give the antigen-binding equation in terms of c_{Ag} (free antigen) and K as

$$\bar{\nu}_{Ag} = \frac{b_{Ag}}{c^0_{Ab}} = \frac{gc_{Ag}K}{1 + c_{Ag}K}. \tag{17}$$

To show that this and other relations are deductions from Eqs. 2 and 14, write the concentration of any complex, c_{1k}, from Eq. 2 by setting $i = 1$ and $f = 1$ and rearranging as

$$\begin{aligned}
c_{1k} &= c^0_{Ab}(rp)^k(1 - rp)^{g-k}g!/[(g - k)!k!], \\
&= c^0_{Ab}(1 - rp)^g[c^0_{Ag}(1 - p)]^k\left[\frac{rp}{(1 - p)(1 - rp)}\right]^k\left[\frac{1}{(c^0_{Ag})^k}\right]\left[\frac{g!}{(g - k)!k!}\right].
\end{aligned} \tag{18}$$

Now substitute Eq. 14 for K to give

$$c_{1k} = c_{10}(c_{01})^k(K)^k\{g!/[(g - k)!k!]\}. \tag{19}$$

This expression for the kth complex in the series of reactions of Eq. 11, with change of symbols, can be seen to be identical with the mass action formula derived by Klotz (1953) and given by Cann (1970, p. 61, Eq. 116) for "protein" (Ab) binding of "ions" (Ag).

The equation given by Cann (1970) for free antibody follows from Eq. 19. In this notation it is

$$c_{Ab} = c_{10} = c^0_{Ab}/(1 + c_{01}K)^g. \tag{20}$$

The average number of Ag bound follows as

$$\bar{\nu}_{Ag} = \frac{c^0_{Ag} - c_{01}}{c^0_{Ab}} = \frac{gc_{01}K}{1 + c_{01}K}, \tag{21}$$

which is seen to be equivalent to Eq. 17. This is not surprising when it is realized that the mass action considerations required the introduction of a "statistical factor." However, it does show that the interpretation of Singer and Campbell is sufficiently general to include hapten binding at a constant K as a special case.

One further relationship between the two theories will be useful. With $f = 1$, Eq. 7 yields

$$\bar{\nu}_{Ag} = grp. \tag{22}$$

Generalization

The Singer-Campbell constant avidity option will be taken to represent those antibody-antigen reactions for which the intrinsic equilibrium constant is constant from antibody excess through antigen excess. With a selected value of K, Eqs. 14 and 15 are used to compute the extent of reaction p.

Verification

Singer and Campbell (1955 *a, b, c*) applied the thermodynamic model to electrophoretic and ultracentrifugal data obtained in antigen excess where there was no precipitation. In order to obtain the concentrations necessary for Eq. 13 when only their sum was available, they made use of a relationship derived from Eq. 10. Data from which a comparison of various methods of computing K can be made have been recomputed and are presented in Table II (Singer and Campbell, 1955 *b, c*). Here, only the region of antigen excess is covered for only two antigens. The agreement for three ways of computing K is quite acceptable over a wide range of avidities and shows consistency of the formulation, but these data also show that K is not a universal constant.

The studies on different mixtures at essentially one pH value (8.4–8.6) are sum-

TABLE II

CONSISTENCY OF THE SINGER-CAMPBELL FORMULATION FOR ALBUMIN-RABBIT IgG SYSTEMS IN ANTIGEN EXCESS AND IN ACID WHERE THE EXTENT OF ANTIBODY REACTION IS LOW (SINGER AND CAMPBELL, 1955 *a*, *b*)

pH	Intrinsic equilibrium constant K computed three ways		
	From free antigen	From free antibody	From soluble complexes*
	liters/mol	*liters/mol*	*liters/mol*
Bovine serum albumin‡			
4.22	3,037	1,530	1,498
3.90	845	849	907
3.88	845	849	808
3.60	215	473	373
3.42	130	267	220
3.31	28	152	146
3.12	130	38	91
Ovalbumins§			
4.29	425	1,735	1,342
4.10	837	1,208	1,063
3.90	425	683	583
3.70	539	574	501
3.50	425	255	276
3.30	674	207	214
3.10	107	125	112

* Ultracentrifugal peak ahead of free antibody taken as sum of AbAg and $AbAg_2$ complexes.

‡ Total concentration, 21.0 mg/ml, $r = 11.7, f = 6, g = 2$, recomputed from given percentage distribution by ultracentrifugation using molecular weights cited by authors (Ag 70,000; Ab, 160,000; Singer and Campbell, 1955 *a*).

§ Total concentration, 14.0 mg/ml, $r = 13.6, f = 5, g = 2$, recomputed from given percentage distribution by ultracentrifugation using molecular weights cited by authors (Ag, 44,000; Ab, 160,000; Singer and Campbell, 1955 *b*).

marized in Table III. There is no obvious trend in the computed values of K (column 3) with increasing r (column 2), and, within the rather large coefficient of variation of 15%, K is constant. The adequacy of these data to prove that K is constant can be challenged as follows.

Singer and Campbell performed their experiments at approximately the same total protein concentration (column 6, Table III) for which a decrease in the ratio of antibody to antigen also involved a decrease in the initial concentration of antibody. Thus, it is not readily apparent if the K they reported as constant is independent of concentration. However, there are two pairs of samples (I-2, VI-2 and

TABLE III

VERIFICATION OF THE SINGER-CAMPBELL CONSTANT AVIDITY OPTION FOR BSA-RABBIT IgG SYSTEM IN ANTIGEN EXCESS AT pH 8.4–8.6 (SINGER AND CAMPBELL, 1955 *c*)

Sample*	r‡	Intrinsic equilibrium constant K§	Variable K options‖		Total concentration
			$gc^0_{Ab}K$	$fc^0_{Ag}K$	
		10^4 liters/mol			*mg/ml*
I	3.6	1.82	2.67	9.7	18.0
II	3.8	0.61	0.89	3.4	18.0
VI	6.3	39¶	38¶	243¶	15.0
I-1	6.9	0.50	0.49	3.3	15.7
VI-2	8.6	0.79	0.66	5.7	15.0
I-2	11.3	0.53	0.52	5.9	20.7
VI-2	11.4	1.41	0.99	11.3	15.0
II-2	14.6	0.61	0.52	7.6	21.3
VI-3	15.3	1.12	0.65	9.9	15.0
VI-4	22.1	0.86	0.38	8.5	15.0
VI-5	34.2	1.14	0.36	12.2	15.0
Mean¶		0.94	0.81	7.8	
Standard error¶		±0.14	±0.22	±1.0	
Coefficient of variation** (%)		15	27	13	

* Specific antibody prepared at pH 7.5, but analyzed at pH 8.43 for samples VI-VI-5 and at pH 8.6 for all others.

‡ Entire table recomputed from given percentage distribution by electrophoresis; r is ratio of total antigen sites to antibody sites using $f = 6$, $g = 2$, and molecular weights cited by authors (Ag, 70,000; Ab, 160,000).

§ Computed from free antigen concentration and used to test for constant avidity.

‖ Two extreme products of K with initial antibody concentration and with initial antigen concentration.

¶ Sample VI omitted in mean and standard error calculations.

** 100 (standard error)/(mean); the smaller the value, the more constant the column of numbers is, provided there is no trend.

II-2, VI-3) of Table III, that might almost be considered dilutions of stock solutions. In both cases, K increased on dilution. One way to check for any trend in all the data is to test two extreme nonconstant K models: the product of K and the initial antibody concentration (column 4) and the product of K and the initial antigen concentration (column 5). Looking at the means and their standard errors, at the bottom of columns 3, 4, and 5, it can be seen that the last model has the smallest variation. However, since the coefficients of variation for all three models are rather large, no definitive statement can be made about the constancy of K with dilution from these data alone. More extensive data are considered next.

HUDSON EXTENSIVE ANTIBODY HETEROGENEITY OPTION

Heterogeneity of Antibody Concept in Hapten Binding

In the development of the hapten-binding equations, the reaction of each antibody site was assumed to be independent of the state of other sites. Even so, each site on the same molecule or each pair of identical sites may have different avidities. Because the haptens are relatively simple compounds and of unit valence, any deviation from Eq. 17 detected in a univalent antigen system has usually been ascribed entirely to such antibody heterogeneity of binding avidities.

If $P(K)$ is the distribution function that describes the heterogeneity of the intrinsic equilibrium constant, $\int_0^\infty P(K)\mathrm{d}K = 1$, then $gc^0_{Ab}P(K)\mathrm{d}K$ is the number of antibody sites per unit volume that have an equilibrium constant between $K - (\mathrm{d}K/2)$ and $K + (\mathrm{d}K/2)$. Karush (1956) assumed that the reaction for each such differential group of sites proceeded independently of every other differential group. Expressed per unit of volume, the total hapten bound would be the sum of the amounts bound by each group. Thus, extending Eq. 17,

$$\frac{\bar{\nu}_{Ag}}{g} = \int_0^\infty \left(\frac{c_{Ag}K}{1 + c_{Ag}K}\right) P(K)\mathrm{d}K = 1 - \int_0^\infty \left(\frac{1}{1 + c_{Ag}K}\right) P(K)\mathrm{d}K. \quad (23)$$

Pauling et al. (1944) suggested that a reasonable description of antibody heterogeneity would be in terms of a gaussian distribution of the free energy of antibody-antigen bonds. Thus, the distribution $P'(\ln K/K_0)$ would be preferred over $P(K)$, where $\int_{-\infty}^{\infty} P'(\ln K/K_0)\ \mathrm{dln}\ (K/K_0) = \int_0^\infty P(K)\mathrm{d}K$. With this transformation, the binding equation becomes (Klotz, 1953; Karush, 1956; Bowman and Aladjem, 1963):

$$\frac{\bar{\nu}_{Ag}}{g} = 1 - \int_{-\infty}^{\infty} \left(\frac{1}{1 + c_{Ag}K}\right) P'(\ln K/K_0)\ \mathrm{dln}\ (K/K_0). \quad (24)$$

Sips (1948) and Bowman and Aladjem (1963) gave Fourier transform procedures for determining P' if $\bar{\nu}_{Ag}$ is given, experimentally or theoretically, as a function of c_{Ag}. The latter authors state that these procedures have never been applied to real data because c_{Ag} has been measured over too narrow a range. The reciprocal approach is to assume a distribution and "see" if experimental data fit. If P' is assumed to be a normal distribution against $\ln (K/K_0)$ with mean at $K = K_0$ and standard deviation $\sigma 2^{-1/2}$, where σ is called the heterogeneity index, the required formula is

$$P' = \frac{1}{\sigma\pi^{1/2}} \exp\left\{-\left[\frac{1}{\sigma}\ln\left(\frac{K}{K_0}\right)\right]\right\}^2. \quad (25)$$

Example of Heterogeneous Antibody in Hapten Binding

Pepe and Singer (1959) made BSA a univalent antigen by coupling one benzenearsonic acid residue covalently to the single sulfhydryl group of the mercaptalbumin derivative. Rabbit antibodies of the IgG class directed against the benzenearsonic acid group were isolated in a pure state. Electrophoretic values were given

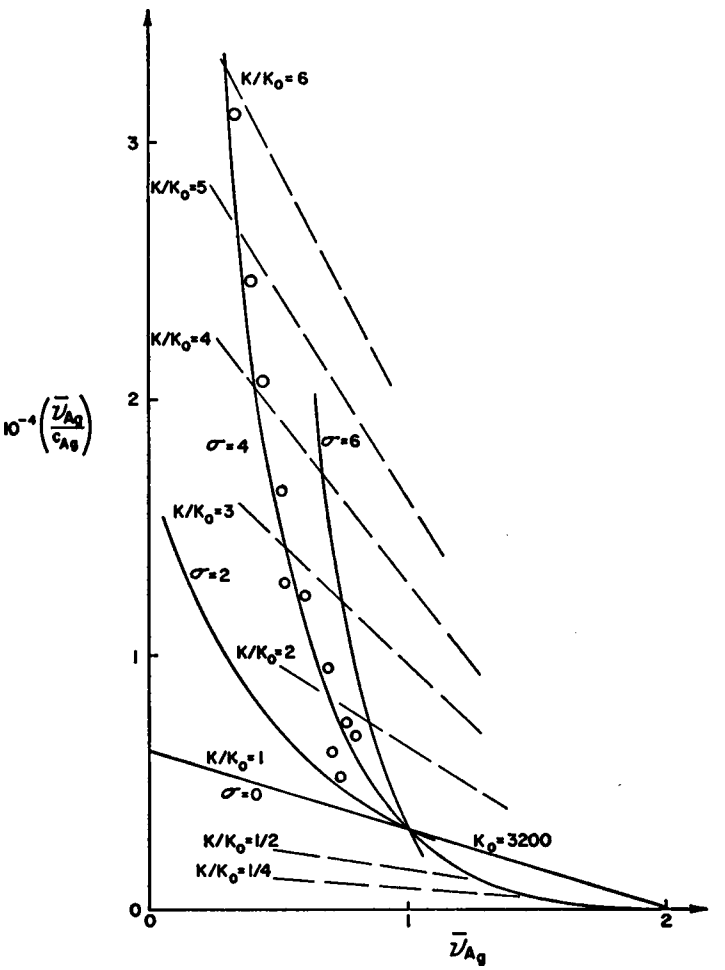


FIGURE 3 Verification of Karush antibody heterogeneity theory of hapten binding in region of high binding. Abscissa and ordinate variables (in molar units) are those for a preliminary binding plot to determine g and K_0. The theoretical curves (solid and dashed) were converted from universal plots of Karush and Sonenberg (1949) using $g = 2$ and $K_0 = 3{,}200$ liters/mol. Experimental points are for benzenearsonic acid-BSA-rabbit IgG system at pH 8.70 (Pepe and Singer, 1959). The antibody concentration varied from 11.5 mg/ml at the upper left to 1.54 mg/ml for the lowest point. The data fit the Karush (1956) theory for a large heterogeneity index of $\sigma = 4$. They cover a sixfold range in the observed apparent intrinsic equilibrium constant.

for the weight concentrations of free antigen and free antibody for known total concentrations of the reagents. Their data can be processed as though from a hapten-binding experiment. The binding plot, shown in Fig. 3, was assumed to extrapolate to $\bar{\nu}_{Ag} = g = 2$. This required drawing an arbitrary curve in the region of high binding, not measured, from which a K_0 of 3,200 liters/mol was calculated from the ordinate corresponding to $\bar{\nu}_{Ag} = 1$. With these values of g and K_0, the general curves for various values of σ were converted from the graph of Karush and Sonenberg (1949). By inspection, it can be seen that these binding data are consistent with the foregoing mathematical model of a very heterogeneous antibody population of $\sigma = 4$. The K/K_0 lines show that there should be about a sixfold variation observed in the apparent equilibrium constant over the range of these electrophoretic data, as was reported and is given in Table IV. These data appear to be the only ones in the literature for varying concentrations of antibody; all equilibrium dialysis studies are reported (and possibly only done) at a single concentration.

TABLE IV

CONSISTENCY OF THE GOLDBERG FORMULATION FOR BENZENEARSONIC ACID BSA-RABBIT IgG SYSTEM AT pH 8.70 OVER ENTIRE RANGE FROM ANTIBODY EXCESS TO ANTIGEN EXCESS (PEPE AND SINGER, 1959)

r*	Intrinsic equilibrium constant K computed three ways			Variable K options§		Total concentration
	From free antigen	From free antibody	From soluble complexes‡	$gc^0_{Ab}K$	$fc^0_{Ag}K$	
	10^4 liters/mol	*10^4 liters/mol*	*10^4 liters/mol*			*mg/ml*
0.197	1.81	2.54	1.92	3.9	0.77	19.4
0.273	1.53	1.51	1.52	2.9	0.78	19.0
0.324	1.31	1.40	1.34	2.4	0.78	18.8
0.452	1.19	0.74	0.98	1.6	0.71	18.3
0.568	0.93	0.77	0.85	1.2	0.57	17.9
0.708	1.07	0.60	0.79	1.0	0.74	17.6
1.06	0.85	0.61	0.69	0.7	0.77	16.9
1.86	0.75	0.50	0.56	0.4	0.76	16.1
2.08	0.57	0.45	0.48	0.3	0.67	16.0
2.49	0.78	0.48	0.54	0.3	0.79	15.8
3.24	0.42	0.42	0.42	0.2	0.65	15.5
			Mean		0.73	
			Standard error		±0.02	

* Entire table recomputed from given percentage distribution by electrophoresis; r is ratio of total antigen sites to antibody sites using $f = 1$, $g = 2$, and molecular weights cited by authors (Ag, 70,000; Ab, 160,000).

‡ Intermediate electrophoretic peak taken as sum of AbAg and $AbAg_2$ complexes.

§ Two extreme products of K, from soluble complexes, with initial antibody concentration and with initial antigen concentration.

This same example can also be used to show the consistency of the concept of extent of reaction even in a heterogeneous system. The results of computing p from the amounts of three different molecular forms present are expressed in terms of K in columns 2, 3, and 4 of Table IV. There is good agreement over the concentration range studied. The rest of the table will be explained below.

Multivalent Antigen Binding

Hudson (1968) used the Farr (1958) technique of additional precipitation of all soluble complexes and free antibody with ammonium sulfate. He computed the

TABLE V

VERIFICATION OF THE HUDSON EXTENSIVE ANTIBODY HETEROGENEITY OPTION FOR BSA-RABBIT IgG SYSTEM IN ANTIGEN EXCESS AT pH 7.5 (HUDSON, 1968)

Dilution*		Fraction as free antigen‡		Intrinsic equilibrium constant K§	Variable K options‖	
					$gc^0_{Ab}K$	$fc^0_{Ag}K$
				10^4 *liters/mol*		
	$r = 1.30$		(1.57 mg/ml)			
1/1		0.1465		3.55	0.585	0.760
	$r = 2.60$		(1.82 mg/ml)			
1/1		0.3650		1.85	0.306	0.793
1/5		0.3615		9.40	0.311	0.806
1/10		0.3585		19.1	0.315	0.817
1/20		0.3705		36.1	0.298	0.773
1/40		0.4355‡		—	—	—
		0.364 ±0.003				0.797 ±0.009
	$r = 5.19$		(2.32 mg/ml)			
1/1		0.6065		0.900	0.149	0.771
1/10		0.6125		8.70	0.144	0.746
1/20		0.6070		17.9	0.148	0.769
1/40		0.6075		35.8	0.148	0.767
1/80		0.6825‡		—	—	—
		0.608 ±0.001				0.763 ±0.006
	$r = 10.4$		(3.32 mg/ml)			
1/1		0.7575‡		—	—	—
1/20		0.7885		8.20	0.068	0.703
1/40		0.7810		17.5	0.072	0.752
1/80		0.7855		33.7	0.070	0.722
1/160		0.8175‡		—	—	—
		0.785 ±0.002				0.726 ±0.014
	$r = 20.8$		(0.133 mg/ml)			
1/1		0.8805		9.20	0.038	0.789
1/2		0.8815		18.1	0.037	0.777
1/4		0.8800		37.1	0.038	0.795
1/8		0.8945‡		—	—	—
		0.881 ±0.001				0.787 ±0.005

TABLE V—*Continued*

Dilution*		Fraction as free antigen‡		Intrinsic equilibrium constant K§	Variable K options‖	
					$gc^0_{Ab}K$	$fc^0_{Ag}K$
				10^4 liters/mol		
	r = 41.5		(0.117 mg/ml)			
½		0.929		12.1	0.025	1.039
¼		0.9375		18.9	0.019	0.808
⅛		0.9260		53.0	0.027	1.136
		0.931 ±0.003				0.994 ±0.097
	r = 83.1		(0.108 mg/ml)			
½		0.9675		9.84	0.010	0.843
¼		0.9640		24.0	0.012	1.031
		0.996 ±0.002				0.937 ±0.094
			Overall mean and standard error			0.820 ±0.025

* Relative dilution factors in each group with ½ referring to total concentrations of reagents given in parenthesis; r is ratio of total antigen sites to antibody sites using $f = 6$, $g = 2$, and molecular weights as cited by author (Ag, 70,000; Ab, 160,000).

‡ c_{0l}/c_{Ag}, determined on supernatant fluid after additional precipitation of free antibody and all soluble complexes with ammonium sulfate (Farr, 1958). All tests, except the first of the r = 41.5 group, were done in duplicate and the values listed are the averages of the values given for an individual serum in Table 3B of Hudson (1968). The values marked are more than 10 standard errors away from the means computed without them and were not used in further calculations. Four of the five discarded values represent every use of a $\frac{1}{160}$ dilution of stock antigen.

§ Computed from free antigen concentration.

‖ Product of K and initial antibody concentration and product of K and initial antigen concentration. Each product is constant in each group, but the second product is constant from group to group.

extent of reaction from the free antigen concentration, as done by Singer and Campbell (1953). His observations on the BSA-IgG system, at pH 7.5 only, led him to postulate that over the entire range from equivalence to extreme antigen excess the system behaved as if the intrinsic equilibrium constant were not actually constant, but rather inversely proportional to the initial total antigen concentration. This means that the product $fc^0_{Ag}K$ was constant not only for experiments at constant c^0_{Ag}, but simultaneously for those at constant c^0_{Ab} and those at any overall dilution. He recognized from Eq. 14 that a constant $fc^0_{Ag}K$ implied a constant extent of reaction, independent of dilution for any particular initial proportion of reagents. He observed that this might either be implicit in Goldberg's theory of antibody-antigen reactions or an expression of extensive antibody heterogeneity. The latter interpretation is chosen here for reasons that will be explained.

The data selected for detailed presentation are those given by Hudson (1968) for an individual serum. His results have been rearranged, in Table V, so that various dilutions are grouped according to the ratio of reactants. The observed average

ratios of free antigen to total antigen are given in the second column. The mean and standard error of the mean in each group are used to test whether values are significantly different within a group or from group to group. For example, the five values marked as footnote‡ are more than 10 standard errors away from the means computed without them and represent mistakes rather than random errors. It appears from column 2 of Table V that the fraction of free antigen remaining is independent of the dilution in each group, but increases as the ratio of reactants goes from $r = 1.3$ to $r = 83.1$.

In the third column of Table V are listed the computed values of the intrinsic equilibrium constant. Note that, for any particular ratio of antigen to antibody, the value of K increased as the system was diluted. Similarly, if the data had been grouped by constant antibody concentration, it would have been apparent for each group that the value of K decreased as the antigen concentration was increased. Hudson's observation that the product $c^0_{Ag}K$ was constant follows from the entries given in the last column of Table V since they have a coefficient of variation of only 3%.

Deviation in Extreme Antigen Excess

The data in Table V were recomputed in terms of antigen binding (Eq. 7) and are shown as the curve with standard error bars in Fig. 4. The abscissa differs considerably from that in hapten binding, and is related to that used by Singer and Campbell (1955 *c*). In the limit as $r \to \infty$ these values of $1/\bar{\nu}_{Ag}$ do not appear to approach $1/g = \frac{1}{2}$ as expected. The explanation, not given by Hudson, can be deduced by considering the rest of his data for individual sera (shown plotted as the labeled curves in Fig. 4).

First, note that the theoretical curve drawn using Eqs. 7 and 14 for $fc^0_{Ag}K = 1$ does not extrapolate to $1/\bar{\nu}_{Ag} = 1/g$. This can be shown, in general, since as $r \to \infty$ $p \to 0$ and $fc^0_{Ag}K = rp/[(1-p)(1-rp)] \to rp/(1-rp)$ or $rp \to 1/[1 + 1/(fc^0_{Ag}K)]$ $\to \bar{\nu}_{Ag}/g$. With K or $gc^0_{Ab}K$ constant, $rp \to 1$, but not so with $fc^0_{Ag}K$ constant. Secondly, viewing the composite of all the data in Fig. 4, an extremely sharp bend at very great antigen excess is evident which, if taken into account, could permit the curves to extrapolate to the correct intercept. Thirdly, this bend explains why three of the sera failed to yield the correct concentration of antibody when their free antigen data were subjected to a proposed least-squares, data-processing procedure based on $c^0_{Ag}K$ = constant (Tables IV and V of Hudson [1968], sera 2–17, 2–28, 2–29).

Extensive Heterogeneity Interpretation

Hudson referred to the data of Pepe and Singer (1959) as also illustrating the constancy of $fc^0_{Ag}K$. This product is given in the sixth column of Table IV and is seen to be relatively constant for these experiments, which were done at roughly a constant total concentration of antibody and univalent antigen, as indicated in the

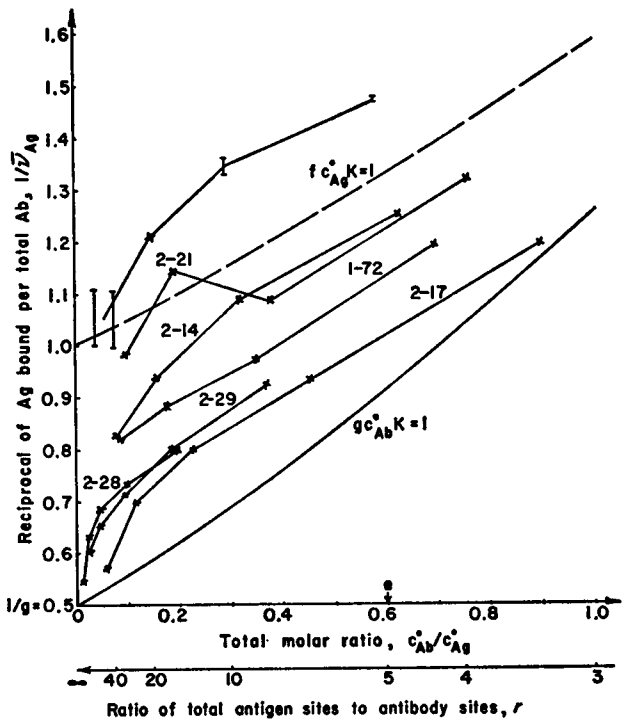


FIGURE 4 Multivalent antigen-binding plot showing need for antigen excess limit for Hudson extensive antibody heterogeneity option. The solid theoretical curve, labeled $gc^0_{Ab}K = 1$, represents a constant K only for a constant antibody concentration, and the dashed curve, labeled $fc^0_{Ag}K = 1$, represents a constant K only for a constant antigen concentration. Curves for constant K with unspecified initial concentrations cannot be drawn. The experimental data are for BSA-rabbit IgG system at pH 7.5, studied over large ranges of concentration, but only the antigen excess region for $r > 3$ was plotted (Hudson, 1968). The number by each experimental curve identifies the individual serum tested. The unlabeled experimental curve at the top with ± one standard error bar corresponds to the groups of Table V. Data in extreme antigen excess show the correct extrapolation to $1/\bar{\nu}_{Ag} = 1/g = 0.5$, but over the rest of antigen excess, the heterogeneity makes $fc^0_{Ag}K$ appear constant. The point e at $r = 5$ represents the inhibition of aggregation limit in antigen excess ($f = 6, g = 2$). The sharp bends in the graphs occur where $r > 25$.

last column. Since these data have already been shown to be describable by a large heterogeneity index of $\sigma = 4$ (Fig. 3), it is tempting to ascribe constancy of $fc^0_{Ag}K$ to extensive antibody heterogeneity. A related inference can be drawn from the work of Karush and Sonenberg (1949). The theoretical hapten-binding plots (their Figs. 3 and 4) represent the reciprocal of the extent of antibody reaction against the reciprocal of the free antigen concentration. For the largest heterogeneity shown ($\sigma = 6$), except for near the origin, the curve is almost horizontal. This means that the extent of reaction is relatively independent of dilution, a requirement if $fc^0_{Ag}K$ is to be constant. However, the result is not universal for the entire

binding curve. Thus, as can be seen from Fig. 3, the region of very high binding is where the data are lacking.

The comparison of heterogeneous hapten binding with Hudson's BSA multivalent binding results is the basis here for claiming that a constant $fc^0_{Ag}K$ represents extensive antibody heterogeneity. Subject to the extreme antigen excess limit, not recognized by Hudson, a constant $fc^0_{Ag}K$ implies that, as the antigen concentration increases, more weakly binding antibodies react and the observed intrinsic equilibrium constant decreases. Similarly, on overall dilution, more weakly bound antibodies dissociate than do strongly bound ones and the observed intrinsic equilibrium constant increases.

Generalization

The remarkable feature of Hudson's observation is that extensive antibody heterogeneity for the multivalent antigens can be described by a simple formula valid over a wide range. The Hudson extensive antibody heterogeneity option will be taken to represent those antibody-antigen reactions for which the antibody heterogeneity is large relative to that of the antigen. The formulation follows from Eqs. 14 and 15 as

$$\begin{aligned} fc^0_{Ag}K &= \text{constant}, \\ r &= fc^0_{Ag}/(gc^0_{Ab}), \\ Q &= (fc^0_{Ag}K)/r, \\ p &= \{1 + Q + rQ - [(1 + Q + rQ)^2 - 4rQ^2]^{1/2}\}/(2rQ). \end{aligned} \quad (26)$$

To make the algorithm complete, a cutoff point in antigen excess must be selected. Beyond that, the extent of reaction is taken as zero and the reactants are considered free. It may be purely academic whether the smallest complex is assumed to be present or whether only free antigen and free antibody exist, because all are likely to be diffusible anyway. Selection of the limit is considered below.

EXTENSIVE ANTIGEN HETEROGENEITY OPTION

Argument by Analogy

In the most probable polymer distribution theory no inherent distinction was made between antigen and antibody. If $fc^0_{Ag}K$ = constant represents a description of extensive antibody heterogeneity with a homogeneous antigen, then, by analogy, $gc^0_{Ab}K$ = constant might represent extensive antigen heterogeneity with a homo-

geneous antibody. The option follows directly from Eqs. 14 and 15 as

$$gc^0_{Ab}K = \text{constant},$$

$$r = fc^0_{Ag}/(gc^0_{Ab}),$$

$$Q = gc^0_{Ab}K,$$

$$p = \{1 + Q + rQ - [(1 + Q + rQ)^2 - 4rQ^2]^{1/2}\}/(2rQ). \quad (27)$$

It is important to note that a cutoff point in antibody excess must be selected, since the constancy of $gc^0_{Ab}K$ prevents the binding from extrapolating correctly as $r \to 0$. This can be seen from Eqs. 7 and 14. For example, as $r \to 0$, $rp \to 0$, and $gc^0_{Ab}K = p/[(1 - p)(1 - rp)] \to p/(1 - p)$ or $p \to 1/[1 + 1/(gc^0_{Ab}K)] \to \bar{\nu}_{Ab}/f$. With K or $fc^0_{Ag}K$ constant, $p \to 1$, but not so with $gc^0_{Ab}K$ constant. The correct extrapolation at the other extreme, $r \to \infty$, is shown for $gc^0_{Ab}K = 1$ in Fig. 4.

Verification

The situation for which data are needed could be envisioned as one where homogeneous antibody cross-reacts with different antigen sites. Perhaps experiments with soluble complexes in antibody excess, say with univalent antibody, could be used to explore antigen heterogeneity.

The work of Klinman (1971) may lead to the availability of the required homogeneous antibody. By selecting only a few antibody-producing cells he obtained homogeneous antibody of a certain avidity. Another "focus" of cells from the same animal also made homogeneous antibody but of a different avidity. Furthermore, he showed that the binding curve for the serum, which contained contributions from all the "monofocal" regions, showed heterogeneity.

GOLDBERG CRITICAL EXTENT OF REACTION OPTION

Precipitation Concept

Goldberg (1952, 1953) made several deductions about the parameter p from the properties of Eq. 2 for m_{ik}. Using summations over all permitted values of i and k, he noted that the expression $1 - rp^2(f - 1)(g - 1)$ occurred in the denominator of an equation for the weight average molecular weight of an aggregate that contained no cyclical complexes. He reasoned that at any initial proportion of antigen to antibody, specified by r, p might increase from zero to a value that would make such a denominator go to zero, which would be mathematically the equivalent of an enormous complex. The "critical" extent of reaction for noncyclical complexes,

p_c, then, would be obtained by setting the above expression equal to zero or

$$p_c = \{1/[r(f-1)(g-1)]\}^{1/2}. \tag{28}$$

Note that this expression is independent of dilution.

Goldberg further reasoned that if the value of p for a complete reaction is less than p_c, then the critical point could not be reached, and the system must be in either extreme antibody or extreme antigen excess regions of the precipitin reaction. In particular, he claimed that the extreme antibody excess region is given mathematically by setting $p_c > 1$, or

$$r < 1/[(f-1)(g-1)], \tag{29}$$

and the extreme antigen excess region is where $p_c > 1/r$ or

$$r > (f-1)(g-1). \tag{30}$$

Correlation of these inhibition of aggregation limits with inhibition of precipitation must involve considerations of solubility of small complexes. Amano et al. (1962) found the same Eq. 28 even with heterogeneity of antigen sites that led to different specific antibodies, provided the total amounts in each of the antibody classes were equal.

Generalization

The Goldberg critical extent of reaction option will be taken to represent the minimum extent of reaction that is either complete or results in a large aggregation of noncyclical complexes. It can be expressed compactly as

$$p = \text{minimum } [1,\ 1/r,\ p_c]. \tag{31}$$

The options that require K pose a problem as to how to select the specific value to use in the simulation. One way is to use the critical extent of reaction at equivalence as a reference value of p and compute from Eq. 14 the corresponding K there for the initial concentration of the reagent originally in the agar. The cutoff limit required in the two heterogeneity options can be selected as any convenient multiple of the appropriate Goldberg limits of Eqs. 29 and 30. For example, in Fig. 4 the deviation from $fc^0_{Ag}K$ = constant occurs for $r > 25$. This is five times the limit given by Eq. 30 when $f = 6$ and $g = 2$.

APPLICATION TO PRECIPITIN ANALYSIS

In order to simulate a quantitative precipitin analysis with the algorithm proposed here it is necessary to set several parameters: (*a*) select the valences, (*b*) select the molecular weights, (*c*) select the concentration or amount of Ab used in the test,

(*d*) select the extent of reaction at $r = 1$, (*e*) select the cutoff limits, and (*f*) specify the complexes that are soluble.

As an example, consider the egg albumin-rabbit pseudoglobulin antibody system at 0.9% NaCl (Aladjem and Lieberman, 1952). These data are shown plotted in Fig. 5 and were chosen because they were simulated by Aladjem et al. (1966) using the extended theory of Palmiter and Aladjem (1963). The computed values for the amount of precipitate at the 10 values of added antigen are plotted in Fig. 5 with the symbol *A*. For their algorithm, they assumed that all complexes with two or fewer Ab were soluble and that both Ab and Ag were homogeneous, except that on each Ag the three sites had a 100-fold range in their intrinsic equilibrium constants.

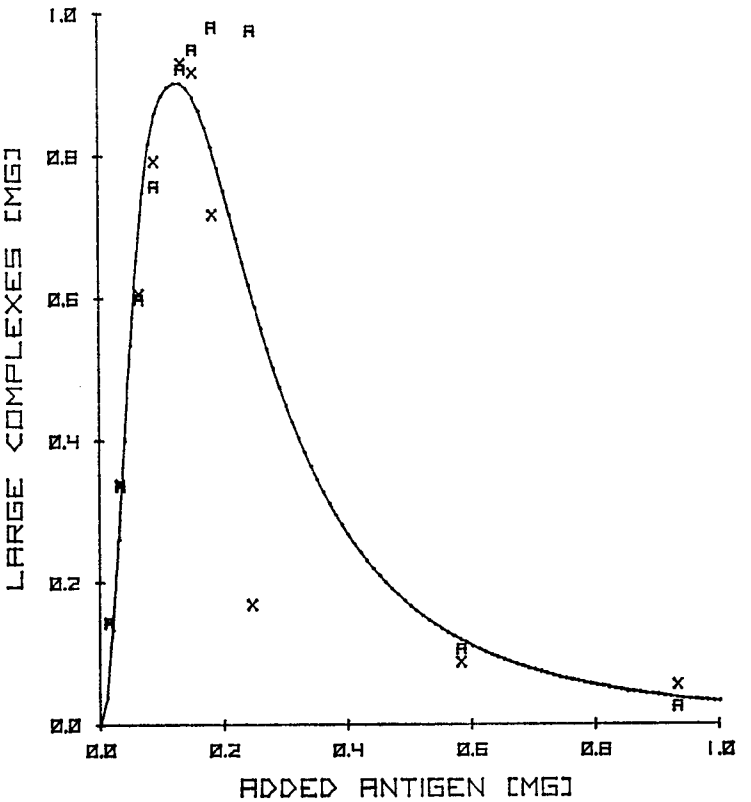


FIGURE 5 Application of algorithm to quantitative precipitin analysis. X, the 10 experimental points of Aladjem and Lieberman (1952) for the amount of precipitate in the egg albumin-rabbit pseudoglobulin IgG system at 0.9% NaCl using a factor of 6.25 to convert milligrams *N* to milligrams. *A*, the computed values of Aladjem et al. (1966), assuming that complexes with two or fewer Ab are soluble. Solid curve, simulation using the algorithm of this paper assuming that complexes with three or fewer Ab are soluble according to the Hudson extensive antibody heterogeneity option with $p = 0.9$ at $r = 1$ (using molecular weights of Ag 30,000 and Ab 160,000).

The solid line in Fig. 5 is the simulation using the extensive Ab heterogeneity option of this paper. Here, all complexes with three or fewer Ab were assumed to be soluble, and the molecular weight of Ag was taken as only 30,000 whereas that of Ab was the usual 160,000 daltons. The value of $fc^0_{Ag}K$ was computed from Eq. 14 by assuming 0.9 for the extent of reaction at $r = 1$. It should be noted that (*a*) the complete reaction and the extensive Ag heterogeneity options yield curves that are quite similar to this one because the extent of reaction is so high; (*b*) since the experiments were performed at a constant Ab concentration, there is no difference between the extensive Ag heterogeneity and the constant avidity options; and (c) the simulated curve for the extensive Ab heterogeneity option is as close to the experimental points as are the points of Aladjem et al. (1966).

DISCUSSION

In radial immunodiffusion, the full range from extreme antibody excess through extreme antigen excess at almost any total concentration level could be encountered somewhere in the gel. In searching for an algorithm for such a dynamic process it was necessary to check each theory for the mathematical consequences of going beyond the supporting experimental data. This includes extrapolation outside the range of concentrations involved and generalization to any valence of antibody or antigen, any strength of reaction, and any kind of heterogeneity. Because simulation of radial immunodiffusion is the ultimate object, it was possible to simplify the search by being concerned mainly with the free reagents and the small complexes. Such an approach made it possible to avoid stringent assumptions about homogeneity, absence of noncyclical complexes, or mechanism of precipitation. Since in simulation the initial amounts of all reagents are known, the amount of large complexes trapped in the gel can be obtained by difference.

The immunochemical reaction was taken as one that produces the most probable distribution of complexes from the interaction of a g-valent antibody with an f-valent antigen. The various theories, even though giving formulas that appear to conflict, have been shown to be interrelated when the extent of reaction is used as a parameter. As a result, the proposal made here is that a general algorithm consists of the Goldberg formula (Eq. 2) for the most probable polymer distribution applied only to free reagents and small complexes. The required extent of reaction parameter p has to be computed first from the initial concentrations of antibody and antigen according to the specific option selected as follows:

(*a*) The Heidelberger-Kendall complete reaction option (Eq. 9). No further parameters are applicable. No equilibrium constants can be computed, the reaction is independent of dilution, there is no free antigen on the antibody excess side of equivalence, and there is no free antibody on the antigen excess side.

(*b*) The Singer-Campbell constant avidity option (Eq. 14). The value of the intrinsic equilibrium constant K for an "isolated" antibody-antigen site reaction is

required. The reaction depends on dilution. This option is for completely homogeneous reagents and permits studies of changes in avidity (say, by pH changes) by selection of different values of K in different simulation trials.

(c) The Hudson extensive antibody heterogeneity option (Eq. 26). The value of the constant product $fc^0_{Ag}K$ is required and a cutoff limit in antigen excess must be specified, such as a multiple of the value given in Eq. 30. The reaction is independent of dilution and is inconsistent with a constant intrinsic equilibrium constant. This option is taken as representing the limiting case of extensive heterogeneity of antibody relative to antigen but where the reaction is nowhere complete. It gives very high extents of reaction in antibody excess and large amounts of small complexes in antigen excess.

(*d*) The extensive antigen heterogeneity option (Eq. 27). The value of the constant product $gc^0_{Ab}K$ is required and a cutoff limit in antibody excess must be specified, such as a fraction of the value given in Eq. 29. The reaction is independent of dilution and is inconsistent with a constant intrinsic equilibrium constant. This option is taken as representing the limiting case of extensive heterogeneity of antigen relative to antibody but where the reaction is nowhere complete. It gives very high extents of reaction in antigen excess and large amounts of small complexes in antibody excess.

(*e*) The Goldberg critical extent of reaction option (Eq. 31). No further parameters are applicable. The reaction is independent of dilution and is inconsistent with a constant intrinsic equilibrium constant. This option provides for more small complexes in the equivalence zone than does the complete reaction. It is useful to provide a value for the minimum extent of reaction that will allow aggregation, probably to the point of precipitation, without cyclical complex formation and to compute cutoff limits for other options.

Unfortunately, it is not readily apparent how the heterogeneity options can be derived from the Amano et al. (1962), Palmiter and Aladjem (1963), and Aladjem and Palmiter (1965) extended theories. This is because Amano et al. do not give a means for determining individual extents of reaction for each site and because Aladjem and Palmiter use implicit integeral equations. The considerations of all these authors, however, do imply that the presence of heterogeneity need not categorically rule out applicability of the Goldberg formulation. Thus, it is proposed here that the two heterogeneity options can be used as limits for real systems: the extensive antibody heterogeneity one supported by experimental data and the extensive antigen heterogeneity one by analogy.

This paper is not intended to be an exhaustive review of antibody-antigen reactions or to redefine "equivalence." Instead, it gives a background as to how formulas were selected for use in the simulation of immunodiffusion. All the formulas presented are deterministic, i.e., the reaction is assumed to achieve the extent of reac-

tion specified by the selected option. Stochastic models in which the actual extent of reaction may be different by chance alone are not considered.

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