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A NEW METHOD OF APPLYING THE SIPS EQUATION

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The Sips equation is frequently used in immunochemistry to describe the relationship between antibody-binding site concentration ($n_o[Ab]$), antigen concentration, intrinsic affinity constant (K), and the heterogeneity index (a) of the affinity constant. Usually $n_o[Ab]$ is determined before calculating the remaining parameters (K and a). A new method is proposed which does not require knowledge of $n_o[Ab]$ nor an extensive calculation to determine K and a. The method can also be used to determine the antibody-binding site concentration without purified antibody or ligand saturation of the binding sites. This method can be applied to any antibody which binds a monovalent ligand and which can be obtained at a concentration greater than $1/K$. Since the Sips equation can be applied to any ordinary chemical reaction by setting $a = 1$, the proposed method can be used generally to determine the affinity constant and the initial concentration of one of the reactants.

Nisonoff and Pressman (1) popularized the use of the Sips equation (2) for the determination of intrinsic affinity and binding constant heterogeneity of antibodies. The form of the equation which they used,

$$r = n_o(KL)^a/[1 + (KL)^a]$$

(see the *Appendix* for definitions),[1] is mathematically tedius but it does not require previous knowledge of the total antibody-binding site concentration, ($n_o[Ab]$). The logarithmic form,

$$\log[r/(n_0 - r)] = a(\log L + \log K)$$

proposed by Karush (3) can be used after $n_o[Ab]$ has been determined by other means. The Farr (4) method (ammonium sulfate precipitation) has been used to measure $n_o[Ab]$, the intrinsic affinity constant (K), and the Sips heterogeneity index (a)(5–8). Equations for use with the Farr method have been proposed by Celada *et al.* (9) for a quantitative expression of antibody avidity (multivalent ligands) and by Paul and Elfenbein (10) for the determination of the intrinsic affinity of homogeneous antibodies or "relative affinity" of hapten-specific heterogeneous antibody preparations. Determination of free ligand concentrations by the Farr method is somewhat controversial (7) because it provides only a very narrow range of accurate measurements. To obtain $n_o[Ab]$, extrapolation from such a narrow range produces a large uncertainty. In the following study, a new method of calculation is proposed which does not require previous knowledge of $n_o[Ab]$ and also uses free ligand values determined in the most accurate range, where half of the total ligand (hapten) is bound to antibody. The proposed method is explained below. A more rigorous derivation is presented in the *Appendix*.

The following example represents an analysis of a hypothetical reaction between a monovalent ligand and monovalent antibody. The relationship is valid in the case of multivalent antibodies as well. The intrinsic binding reaction is represented as:

$$S + L \rightleftarrows SL$$

where $[S]$ = free antibody binding site concentration; $[L]$ = free ligand (hapten) concentration; $[SL]$ = concentration of bound ligand or occupied antibody sites; A = total antibody binding site concentration = $[S] + [SL] = n_o[Ab]$; H = total ligand (hapten) concentration = $[SL] + [L]$; and $K = [SL]/[S][L]$ = intrinsic affinity constant. The "midpoint" (A_m) of the reaction is defined as that antibody-binding site concentration (A) where half of the ligand is bound. At such a midpoint,

$$[SL] = [L] = H/2$$

and

$$K = 1/[S].$$

Then,

$$A_m = H/2 + 1/K. \quad (1)$$

Generally, ligands and antibodies are used at concentrations of 10^{-5} M to 10^{-8} M. To express this wide range, but small quantity, the Sips form of equation 1 (*Appendix*, eq. 2) is plotted on a negative log scale for a hypothetical heterogeneous antibody population (Fig. 1). In this plot, a change in slope divides the curve into two regions so that extrapolation of each slope forms a point of intersection (H_i). To the left of the intersection point, H is much larger than $1/K$. From equation 1, the midpoint antibody-binding site concentration $[A_m]$ is then approximately equal to $H/2$. The curve in this region is a straight line with a slope of one. To the right of the intersection point, H is much smaller than $1/K$. The exact relationship is shown in the *Appendix*.

A hypothetical solution containing two antibody populations with different K's is represented in Figure 2. At a high concentration of $H(H >> 1/K_{low})$, the subset with low K will bind ligand as well as the antibodies with high K. The apparent antibody concentration would be the sum of the concentrations of the two populations. However, at a low concentration of H ($H << 1/K_{high}$), only the subset with high affinity will be able to bind ligand approximating the curve for the antibody population with a high K. The apparent concentration of anti-

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[1] Abbreviations used in this paper: $[Ab]$, concentration of antibody molecules; n_o, number of binding sites per antibody molecule; $n_o[Ab]$, total antibody-binding site concentration; DNP and TNP, the 2,4-dinitrophenyl and 2,4,6-trinitrophenyl determinant groups, respectively; DNP-HOP-lysine, N^{ϵ}-DNP-N^{α}-hydroxyphenyl-L-lysine; TN-8, purified rabbit anti-2,4,6-trinitophenyl antibody; GPaTNP, guinea pig anti-TNP antiserum.

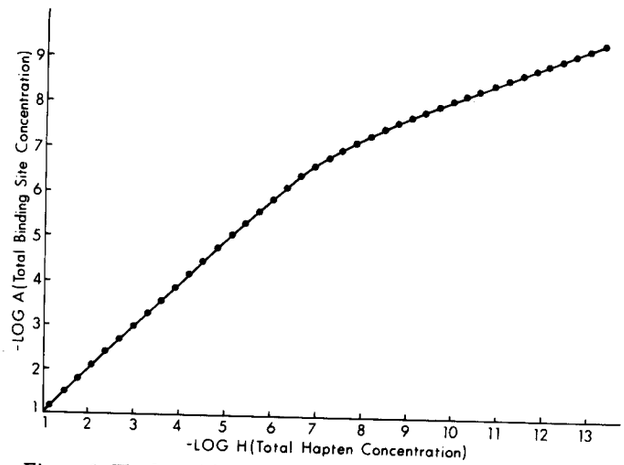

Figure 1. The logarithmic form of equation 2 in the *Appendix* was plotted for a hypothetical heterogeneous antibody population binding a univalent ligand. Since $F = 1$, all A values $= Am$. Assumed values were $K = 10^7\ M^{-1}$, and $a = 0.7$. The calculation was performed on a Hewlett-Packard calculator, model 9815A (Hewlett-Packard Co., Colo.). The program will be provided upon request.

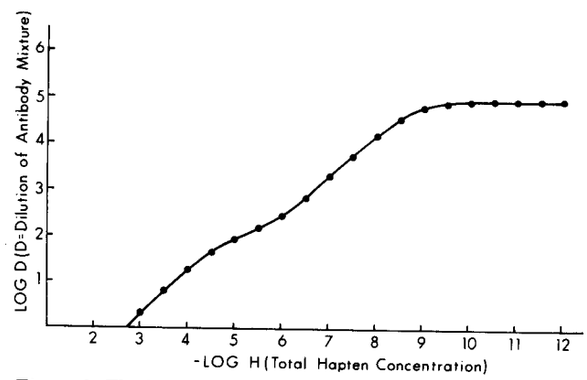

Figure 2. The logarithmic form of equation 2 in the *Appendix* was plotted with midpoints for a hypothetical solution containing two antibodies. It was assumed that the first antibody concentration was 10^{-4} M with $K_1 = 10^9\ M^{-1}$ and $a_1 = 1$. The second antibody concentration was assumed to be 10^{-3} M with $K_2 = 10^5\ M^{-1}$, and $a_2 = 1$. The two nonlinear portions of the curve appear at 9 and 5 on the X axis corresponding to each K value. It is evident that the low affinity antibody would be invisible to any binding assay if the antibody concentrations were 100-fold more dilute. The calculation was performed on a Hewlett-Packard 9815A calculator.

bodies in this region would be that of the high affinity molecules since the low affinity antibody population would be excluded from the reaction. At intermediate ligand concentrations ($1/K_{low} > H > 1/K_{high}$), the curve would show a smooth transition with the exact slope depending on the ratio of relative concentrations of the two subsets of antibodies.

The approach illustrated graphically in Figure 1 can be used to approximate the actual distribution of equilibrium constants among antibodies in serum. In fact, for the Sips distribution of K's with a heterogeneity index, a, the curve to the right of the intersection point will be a straight but continuously rising line with slope equal to $1 - a$ (*Appendix*, eq. 4 and Fig. 1. To apply equation 1 to experimental data, antibody midpoint concentrations (A_m) are determined by a series of Farr assays with different hapten concentrations. When H is much smaller than $1/K$, the slope of the line, ($1\text{-}a$), permits the calculation of a. In the region where H is much greater than $1/K$, the slope of the curve is 1 (*Appendix* eq. 3). Extrapolation of these two lines to a point of intersection (H_i) permits the calculation of K ($H_i = 2/K$, *Appendix*, eq. 5). The serum dilution at the intersection point can be determined graphically (Fig. 1). The antibody-binding site concentration in serum before dilution (A_o) equals ($1/K$) $\times$ (dilution factor), (*Appendix*, eq. 6). This relationship implies that the lowest K measurable by this method is the reciprocal of the binding site concentration.

In summary, the determination of midpoints where half the total ligand is bound to antibody permits the determination of the three parameters in the Sips equation: antibody-binding site concentration, intrinsic affinity constant, and the heterogeneity index.

MATERIALS AND METHODS

To establish the validity of this new method, experiments were performed following a protocol adapted from Stupp *et al.* (5). Four polynitrophenyl-specific (DNP or TNP) antibodies were used, namely: serum of mice bearing plasmacytoma MOPC 315 (kindly provided by Dr. H. N. Eisen), purified 315 protein (11), purified rabbit anti-2,4,6-trinitrophenyl antibody (TN-8), and guinea pig anti-2,4,6-trinitrophenyl antiserum (GPaTNP). Both TN-8 and GPaTNP are known to cross-react with DNP (12). ^{3}H-DNP-L-lysine was either prepared (13) or purchased from New England Nuclear, Boston, Mass. (1.1 Ci/mmole). N^{ϵ}-DNP-N^{α}-hydroxyphenyl-L-lysine (DNP-HOP-lysine, kindly provided by Dr. John Freed) was iodinated by the chloramine-T method (14).

Three-fold serial dilutions of antiserum or purified antibody were made in phosphate-buffered saline (0.02 M potassium phosphate, 0.15 M NaCl, pH 7.4) with 10% heat-inactivated normal rabbit serum or in Tris-HCl (0.1 M, pH 7.5) (Sigma Chemical Co., St. Louis, Mo.) containing 1 mg/ml rabbit IgG (Fraction II, Miles Laboratories, Inc., Kankakee, Ill.). These dilutions ranged from $1:3^0$ (undiluted) to $1:3^{10}$ and included an infinite dilution ($1:3^{\infty}$) containing only buffer solution.

To determine each midpoint, 50 μl of a selected concentration of ^{3}H-DNP-L-lysine were added to 50 μl aliquots of each antibody (or antiserum) dilution in micro test tubes (Brinkman Instruments, Inc., Westbury, N. Y.). These were mixed and incubated at 4°C for 15 to 60 min. Then 100 μl saturated ammonium sulfate solution (4°C) were added, mixed, and incubated 30 min to 18 hr at 4°C. Such variations in incubation times did not affect the resultant midpoint (unpublished results). The mixture was centrifuged at 4°C and 12,000 $\times$ G for 2 min. Then 100 μl of the supernatant were added to 10 ml of a solvent for liquid scintillation counting (5). The radioactivity was measured in a Packard liquid scintillation spectrometer (Packard Inst. Co., Inc., Downers Grove, Ill.). To achieve a wide range of hapten concentrations, the specific activity of the ^{3}H-DNP-L-lysine was varied by addition of ^{1}H-DNP-L-lysine. For each hapten concentration the supernatant cpm were plotted against the corresponding antibody dilution to determine the midpoint (A_m).

A slightly different procedure was used for binding studies with 315 protein and ^{125}I DNP-HOP-lysine. Tris-HCl (0.1 M, pH 8) was used for the dilution of ^{125}I-DNP-HOP-lysine and 0.7 mg/ml of rabbit IgG was added as the carrier protein. The ^{125}I in the sample supernatant (100 μl) was counted directly in a Packard Auto-Gamma scintillation counter.

RESULTS

The relationship between supernatant cpm and GPaTNP antiserum dilutions at different hapten concentrations is illustrated in Figure 3. The antibody dilution at the middle of each transition (the first order approximation) is taken as the midpoint antibody dilution, as shown by the arrows on each curve (Fig. 3).

To determine the reproducibility of this method, four separate experiments were performed with GPaTNP antiserum and ^{3}H-DNP-L-lysine (Fig. 4). One set of lines was drawn to fit these data points by linear regression analysis. When $H >> 1/K$, the slope of the line was 0.845 (correlation coefficient = 0.994, standard error of estimate = 0.050). When $H << 1/K$ (to the right of the intersection point), the slope was 0.288 (correlation coefficient = 0.793, S.E. of estimate = 0.085). The slope of the curve to the left of the intersection point (0.845) is different from the theoretical value of 1.0 and may be due to very weak binding of ligand by high concentrations of serum albumin. The greater dispersion of midpoints at very dilute hapten concentrations was accounted for by the relatively low specific activity of the ligand and the resulting low cpm in these supernatant samples. The linear regression analysis of the combined data in Figure 4 gave an affinity constant of 7.8×10^7 M^{-1}. However, average values and standard deviations of the binding parameters were calculated from the four separate experiments: the average affinity constant (K) was $10.2 \pm 3.7 \times 10^7$ M^{-1}, the heterogeneity index (a) was 0.68 ± 0.07 and the total antibody concentration [Ab] was 0.62 ± 0.18 mg/ml (assuming two binding sites per antibody molecule and a m.w. of 150,000 daltons for guinea pig IgG). The variance of K was calculated by analysis of the expectations of functions of random variables (16). Log $K \pm$ S.E. was 7.9 ± 1.1. The same data plotted in Figure 4 were used to calculate K by a traditional method (17) based on the assumption that the ligand titrations provided a reliable value for the bivalent antibody concentration of each sample. With this method, the average values ($\pm$ S.D.) were: $K = 4.7 \pm 3.0 \times 10^7$ M^{-1} and $a = 0.76 \pm 0.07$. These values are in satisfactory agreement with independent determinations (unpublished) of antibody concentration in serum (0.91 mg/ml) determined by quantitative precipitin analysis and of K (6.0×10^7 M^{-1}) and a (0.63) determined by antibody fluorescence quenching by using antibodies isolated from the same antiserum by specific precipitation and hapten elution (12).

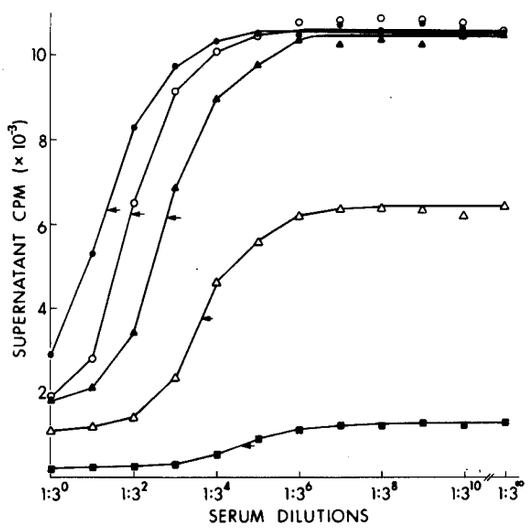

Figure 3. The relationship between supernatant cpm and antiserum dilutions illustrates the basic method for determining "midpoints." The curves are data from one experiment with GPaTNP binding ^{3}H-DNP-L-lysine. The arrows mark the serum dilution that lies at the midpoint of each transition. Data for five concentrations of ^{3}H-DNP-L-lysine (total ligand) are shown: ● = 1.62×10^{-6} M, ○ = 7.28×10^{-7} M, ▲ = 2.74×10^{-7} M, △ = 7.63×10^{-8} M, and ■ = 1.53×10^{-8} M. The total ligand concentrations are the final concentrations after the addition of $(NH_4)_2SO_4$. Lower concentrations of hapten were also used in each experiment, but were plotted on expanded coordinates (not shown).

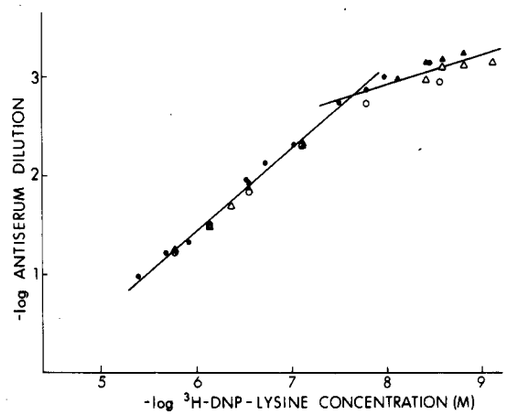

Figure 4. Midpoint values for GPaTNP binding ^{3}H-DNP-L-lysine from four different experiments. The assays were performed on different days with independently prepared ligand stock solutions and antiserum dilutions to evaluate the reproducibility of the proposed method. The binding parameters and statistical analysis are given in the text.

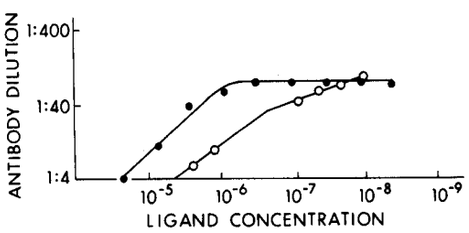

Figure 5. Plot of midpoints for experimental data obtained for the binding of ^{3}H-DNP-L-lysine by isolated 315 protein (●) and by isolated rabbit anti-TNP antibody from serum pool, TN-8 (○). The abscissa gives molar ligand concentrations. The values for K, a and [Ab] calculated from these data are given in Table I.

The midpoint values for MOPC 315 serum and isolated TN-8 are shown in Figure 5. The parameters determined from these experiments are compared with reported values in Table I. The affinity constant of the purified 315 protein for DNP-HOP-lysine is lower than the value reported for ϵ-DNP-L-lysine (10, 11) which is probably related to the presence of the hydroxyphenyl moiety on the α amino group. The binding site concentration is expressed as molar concentration (M) and in mg/ml for the purpose of comparison with literature values assuming two binding sites per Ig molecule and m.w. of 150,000 daltons. Also shown in Table I are the binding parameters determined by the proposed new method of 315 protein isolated from serum as described elsewhere (11, 15).

TABLE I

Independent determination of antibody-binding parameters

	Measured Values				Values in the Literature			
	Binding site concentration[a]	Ig concentration[a]	K (M^{-1})	a^b	Ig concentration[a]	K (M^{-1})	a^b	Reference
	M	*mg/ml*			*mg/ml*			
Isolated TN-8	0.7×10^{-5}	0.5	4×10^6	0.6	0.53	6.3×10^6	0.58	(12)
MOPC 315 serum[c]	1×10^{-4}	7	2×10^6	1	6	5×10^6	1	(10, 15)
Isolated 315 protein	5×10^{-5}	3.7	6×10^6	1	3.5	5×10^6	1	(10, 15)

[a] The concentration of the purified antibody was measured by the optical density at 278 nm. The amount of the 315 protein in pooled serum from tumor-bearing BALB/c mice was determined by quantitative precipitin reaction with $DNP_{10}HSA$ as antigen.

[b] Sips heterogeneity index.

[c] The ligand used for the binding studies with MOPC 315 serum was ^{125}I-DNP-HOP-lysine. The other binding studies were performed with ^{3}H-DNP-L-lysine, and the values quoted from the literature were obtained with DNP-L-lysine.

DISCUSSION

When the three parameters of ligand-antibody reactions are determined without any extrapolation from an accurate but narrow range of measurements, the Farr method produces acceptable results without any paradox (7). However, the new method proposed in this work has several advantages. First, it utilizes the simplicity of the Farr technique but enhances its accuracy by using only the midpoints where half the total ligand is free. Second, the antibodies need not be purified from the serum. Third, it is not necessary to know the antibody-binding site concentration; only the ligand concentration must be known. Fourth, this method does not require extensive calculations. Furthermore, the assay can be performed on a microscale. In many cases, the entire experiment may be performed with less than 0.5 ml of undiluted serum and in some cases, less than 0.1 ml of the serum was required. Of course, this may not be possible if there are only extremely small amounts of antibody in the serum or if the antibody has low affinity (K). This method would be applicable for a radioimmunoassay where only one parameter, i.e., ligand concentration, need be determined but it is limited to the use of monovalent ligands and to antibody concentrations greater than $1/K$.

This proposed method of data analysis is similar to the empirical expressions derived by Celada *et al.* (9) and used by them and others for the calculation of antibody avidity. However, the rigorous mathematical analysis of antibody reactions with multivalent antigens is extremely complex and, as noted by Paul and Elfenbein (10), the Celada equation does not provide an unambiguous value for avidity even when univalent ligands are used for binding studies with heterogeneous antibody populations. The method of analysis proposed by Paul and Elfenbein (10) is preferable for the determination of relative affinities but it does not provide for the calculation of antibody concentrations in antisera, nor can it be used to obtain average intrinsic association constants for univalent ligand binding by populations of antibodies displaying affinity heterogeneity. The method proposed here for the calculation of antibody average affinity expresses the molar ligand concentrations and antiserum dilutions existing after the addition of $(NH_4)_2SO_4$, as suggested by Seppälä (18).

Werblin and Siskind (19) have provided evidence that asymmetrical and multimodal distributions of affinities often occur in anti-hapten antisera. For the detailed analysis of complex binding reactions of heterogeneous antibodies, the Sips equations are oversimplifications, however, alternative methods of calculations (20) are much more laborious than those presented here and require relatively large amounts of purified antibodies or immunoglobulin fractions.

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APPENDIX

Derivation of the relations presented in the paper:

$$r = n_o(KL)^a/[1 + (KL)^a]$$

can be converted into

$$F = r[Ab]/L = AK^aL^{a-1}/[1 + (KL)^a] \quad (1)$$

where the variables are, r = moles ligand bound/mole antibody, n_o = number of binding sites per antibody molecule, K = intrinsic affinity constants, L = free ligand concentration, a = Sips heterogeneity index, F = bound ligand/free ligand, $[Ab]$ = concentration of antibody molecules, A = total antibody binding site concentration = $n_o[Ab]$, H = total ligand concentration.

Set $F = 1$, then equation (1) becomes

$$1 + (KL)^a = AK^aL^{a-1}, \text{ and } (KL)^{-a} = A/L - 1. \quad (2)$$

If $KL \gg 1$, then

$$A/L - 1 \simeq 0$$

$$A \simeq L = H/2, \text{ at } F = 1$$

$$(d/d \log H)(\log A) \simeq 1. \quad (3)$$

If $KL \ll 1$, (i.e., $KL \simeq 0$), then

$$AK^aL^{a-1} \simeq 1.$$

$$\text{Since } L = H/2, \text{ at } F = 1,$$

$$(d/d \log H)(\log A) \simeq (1 - a). \quad (4)$$

At the intersection point,

$$L_i = H_i/2 = 1/K \quad (5)$$

$$A_o = DH_i/2 = D/K \quad (6)$$

where D is the antiserum dilution factor, H_i is the ligand concentration at the intersection point, and A_o is antibody binding site concentration before dilution.

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