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Theory of Heterogeneity in Displacement Reactions

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We investigate the effects of a heterogeneous distribution of affinity constants on association/dissociation and displacement reactions. Through a theoretical analysis, we show that the low-concentration limit of the reaction isotherm depends exclusively on the high-affinity tail of the distribution. If the high-affinity tail decays rapidly, the reaction follows the standard Langmuir isotherm. However, if the high-affinity tail decays slowly, the form of the isotherm crosses over to the Sips isotherm. The threshold for this crossover is different for association/dissociation and displacement reactions. These results have broad implications for the assessment of heterogeneity in displacement assays.

Chemical reactions often occur in heterogeneous systems. One common example in surface chemistry is the adsorption of gas atoms onto a heterogeneous substrate. The sites of the substrate have, in general, a distribution of adsorption energies and, hence, a distribution of affinities for the gas atoms. Heterogeneity is even more common in biological reactions, such as the binding of antigens onto antibodies. Antibodies differ from each other in molecular structure and in thermal fluctuations. They therefore have a distribution of affinities for antigens. In these and related systems, one must investigate how the heterogeneous distribution of affinities affects the equilibrium state of a chemical reaction.

This problem of heterogeneity was first considered in the context of surface adsorption. Surface adsorption can be described by the association/dissociation reaction $A + B \rightleftharpoons AB$, where A represents gas atoms and B represents adsorption centers on a solid substrate. In a homogeneous system, this reaction is described by the Langmuir isotherm. For low concentrations [A], the Langmuir isotherm becomes

$$[AB] \propto [A] \tag{1}$$

However, experimental studies of adsorption onto heterogeneous substrates have instead found the isotherm

$$[AB] \propto [A]^a \tag{2}$$

where a is an exponent between 0 and 1. To understand this anomalous power law, Halsey and Taylor[1] considered possible forms for the affinity distribution and derived the resulting forms for the adsorption isotherm. Through this modeling procedure, they found an affinity distribution that generates an isotherm in agreement with eq 2.

In a further theoretical study, Sips[2] considered the opposite mathematical problem for the same chemical system. He began with a specific expression for the adsorption isotherm, which reduces to eq 2 in the low-concentration limit, and used complex analysis to determine the unique affinity distribution that generates this isotherm. He showed that this affinity distribution resembles a Gaussian distribution (although it differs from a Gaussian in a crucial way, discussed below). In his theory, the exponent a can be interpreted as an index of heterogeneity, which is related to the width of the affinity distribution. The limit $a = 1$ corresponds to a homogeneous system, while $a < 1$ corresponds to increasing heterogeneity.

The Sips approach has become a standard method for assessing heterogeneity in immunological systems.[3−9] In an antibody−antigen binding reaction, A represents an antigen and B the corresponding antibody. Experimental measurements of the reaction isotherm are fit to the power law of eq 2. The resulting exponent a is interpreted as an index of heterogeneity for a system of antibodies.

The purpose of this paper is to re-examine the assumptions that go into the Sips analysis. We address the following questions: How general is the Sips analysis? Does it apply only to a particular distribution of affinities or to a broader class of distributions? What is the physical interpretation of the heterogeneity index a? Most importantly, does the Sips analysis still apply if one has a *displacement* rather than an *association/dissociation* reaction, as is often the case in studies of antibody−antigen binding? Through this theoretical study, we determine when the Sips analysis is valid and when it must be modified.

(1) Halsey, G.; Taylor, H. S. *J. Chem. Phys.* **1947**, *15*, 624−630.
(2) Sips, R. *J. Chem. Phys.* **1948**, *16*, 490−495.
(3) Nisonoff, A.; Pressman, D. *J. Immunol.* **1958**, *80*, 417−428.
(4) Nisonoff, A.; Pressman, D. *J. Immunol.* **1958**, *81*, 126−135.
(5) Karush, F. *Adv. Immunol.* **1962**, *2*, 1−40.
(6) Karush, F.; Karush, S. S. In *Methods in Immunology and Immunochemistry*; Williams, C. A., Chase, M. W., Eds.; Academic Press: New York, 1971; Vol. 3, pp 389−393.
(7) Nahm, M. H.; Herzenberg, L. A.; Little, K.; Little, J. R. *J. Immunol.* **1977**, *119*, 301−305.
(8) Berzofsky, J. A.; Epstein, S. L.; Berkower, I. J. In *Fundamental Immunology*, 2nd ed.; Paul, W. E., Ed.; Raven Press: New York, 1989; pp 315−355.
(9) Olson, W. C.; Colton, C. K.; Yarmush, M. L. *J. Membr. Sci.* **1991**, *56*, 247−278.

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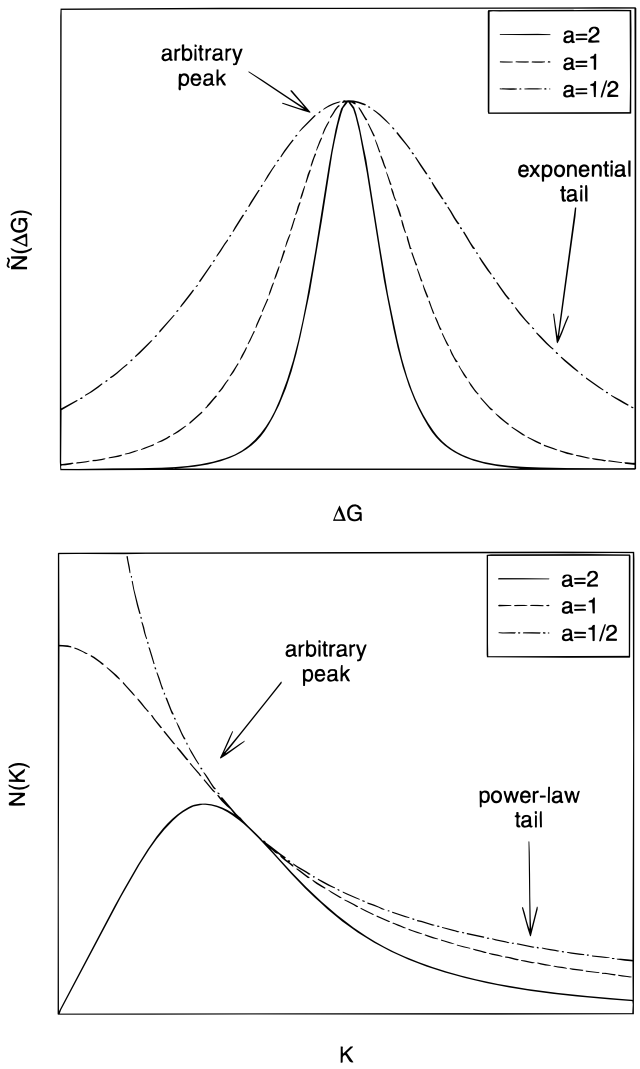

Figure 1. (a) Heterogeneous distribution $\tilde{N}(\Delta G)$ of the binding free energy ΔG for a reaction. The bulk of the distribution may have any arbitrary form, but the tail is assumed to decay exponentially as $\tilde{\alpha}e^{-a\Delta G/k_BT}$. The graph shows $a = 2$ (solid line), $a = 1$ (dashed line), and $a = {}^1/_2$ (dot−dashed line). (b) The corresponding distribution $N(K)$ of the affinity $K = K_0e^{\Delta G/k_BT}$. The bulk of the affinity distribution has an arbitrary form, but the tail decays as the power law αK^{-a-1}. Again, the graph shows $a = 2$ (solid line), $a = 1$ (dashed line), and $a = {}^1/_2$ (dot−dashed line).

The plan of our paper is as follows. First, we derive the isotherm for any association/dissociation reaction in the low-concentration limit. We show that the form of the isotherm depends only on the high-affinity tail of the affinity distribution. If this tail decays rapidly, the reaction follows the Langmuir isotherm (eq 1), but if it decays sufficiently slowly, the reaction follows the Sips isotherm (eq 2). The heterogeneity index a then has a very simple physical interpretation in terms of the tail of this distribution. Next, we apply the same mathematical analysis to a displacement reaction. We find that the Sips isotherm also applies to a displacement reaction, but the threshold for the crossover to the Sips form is different. Finally, we discuss these results in the context of displacement assays of antibody−antigen binding. The distinction between association/dissociation and displacement reactions turns out to be essential for the assessment of heterogeneity in displacement assays.

ASSOCIATION/DISSOCIATION REACTIONS

In this section, we consider an arbitrary association/dissociation reaction of the form

$$A + B \rightleftharpoons AB \tag{3}$$

In surface adsorption, A represents gas atoms, which are present in a low concentration that can be controlled experimentally, and B represents adsorption centers on a solid substrate. In typical antibody−antigen binding reactions, A represents antigens, which are introduced in low concentrations, and B represents the corresponding antibodies. In general, A represents the reactant whose concentration can be controlled experimentally, while B represents the other reactant. In the rest of this section, we will use the antibody−antigen terminology.

For the association/dissociation reaction (eq 3) the law of mass action states

$$[AB]/[A][B] = K \tag{4}$$

where K is the affinity constant, which is related to the binding free energy ΔG by $K = K_0e^{\Delta G/k_BT}$. It is convenient to define the reaction coordinate

$$r \equiv [AB]/([B] + [AB]) \tag{5}$$

This reaction coordinate represents the fraction of antibodies B that are bound to antigens A. It corresponds to the surface coverage θ in a surface adsorption experiment.[1,2] In terms of this reaction coordinate, the law of mass action can be written as

$$r = K[A]/(1 + K[A]) \tag{6}$$

This equation is known as the Langmuir isotherm. For low concentration [A], it reduces to the linear scaling of eq 1.

Now suppose that a system contains a heterogeneous mixture of antibodies. This mixture can be characterized by a distribution $\tilde{N}(\Delta G)$ of the binding free energy ΔG, as shown in Figure 1a. The bulk of the distribution may have any arbitrary form. We assume only that the tail of this distribution above the threshold ΔG_C decays exponentially: For $\Delta G \gtrsim \Delta G_C$, the distribution becomes

$$\tilde{N}(\Delta G) = \tilde{\alpha}e^{-a\Delta G/k_BT} \tag{7}$$

As an alternative description, the mixture can be characterized by the distribution $N(K)$ of the affinity K, as shown in Figure 1b. The distribution of affinity is related to the distribution of binding free energy by $N(K)\,dK = \tilde{N}(\Delta G)\,d(\Delta G)$. Hence, our assumption (eq 7) implies that the tail of the affinity distribution above the threshold $K_C = K_0e^{\Delta G_C/k_BT}$ decays as a power law: For $K \gtrsim K_C$, it becomes

$$N(K) = \alpha K^{-a-1} \tag{8}$$

where $\alpha = \tilde{\alpha}k_BTK_0^a$. We must have $a > 0$ so that the integral of the distribution is finite. Note that the tail of our distribution is equivalent to the Halsey−Taylor distribution,[1] and to the tail of

the Sips distribution,[2] but we now leave the rest of the distribution as an arbitrary function.

To obtain the overall fraction of antibodies that are bound to antigens, we must integrate over the affinity distribution. This integration gives

$$r = \int_0^\infty \mathrm{d}K N(K) \frac{K[\mathrm{A}]}{1 + K[\mathrm{A}]} \tag{9}$$

To derive the leading behavior of this integral for low concentrations [A], we factor out an overall factor of [A] and break up the integral as

$$r = [\mathrm{A}]\left[\int_0^{K_\mathrm{C}} \mathrm{d}K N(K) \frac{K}{1 + K[\mathrm{A}]} + \int_{K_\mathrm{C}}^\infty \mathrm{d}K \alpha K^{-a-1} \frac{K}{1 + K[\mathrm{A}]}\right] \tag{10}$$

In the limit of low [A], the first integral is just a finite constant with respect to [A]. The behavior of the second integral depends on the value of a. If $a > 1$, this second integral is also a finite constant with respect to [A]. In that case, for low [A], the sum of the integrals gives

$$r = \bar{K}[\mathrm{A}] \tag{11}$$

where $\bar{K} = \int_0^\infty K N(K)\, \mathrm{d}K$ is the mean affinity. This expression is the low-concentration limit of the Langmuir isotherm (eq 1). By contrast, if $a < 1$, the second integral diverges as [A] → 0. For sufficiently small [A], the first integral is negligible compared with the second. In this case, eq 10 becomes

$$r = [\mathrm{A}] \int_{K_\mathrm{C}}^\infty \mathrm{d}K \alpha K^{-a-1} \frac{K}{1 + K[\mathrm{A}]} = \alpha[\mathrm{A}]^a \int_{K_\mathrm{C}[\mathrm{A}]}^\infty \mathrm{d}k \frac{k^{-a}}{1 + k} \tag{12}$$

where $k = K[\mathrm{A}]$. In the limit [A] → 0, the lower limit of this integral goes to zero, and the integral can be evaluated explicitly as

$$r = \frac{\pi\alpha}{\sin \pi a}[\mathrm{A}]^a \tag{13}$$

This expression is the Sips isotherm (eq 2). In logarithmic form, these results can be summarized as

$$\log r = \begin{cases} \log [\mathrm{A}] + \log \bar{K} & \text{for} \quad a > 1 \\ a \log [\mathrm{A}] + \log\left[\dfrac{\pi\alpha}{\sin \pi a}\right] & \text{for} \quad 0 < a < 1 \end{cases} \tag{14}$$

To interpret these results physically, note that the *low-concentration* limit of the binding isotherm is dominated by the high-affinity tail of the affinity distribution. This result is reasonable: If the antigens are rare, then only the highest affinity antibodies will bind to them. The exponent a describes the distribution of the highest affinity antibodies. If $a > 1$, then this distribution decays relatively quickly, and the low-concentration limit of the binding isotherm is described by standard Langmuir form, with the mean affinity $\bar{K}$. However, if $a < 1$, then there are many antibodies with extremely high affinities. As the concentration [A] approaches 0, the binding is dominated by antibodies farther out on the affinity distribution. For that reason, the binding isotherm crosses over to the nonanalytic Sips form. The value $a = 1$ is the threshold for this crossover.

In his original paper, Sips[2] found a specific form for the distribution $\tilde{N}(\Delta G)$ of the binding free energy ΔG, which implies a specific form for the affinity distribution $N(K)$. He argued that this distribution resembles a Gaussian distribution. However, as Goldstein[10] pointed out, the Sips distribution only resembles a Gaussian near the peak. In the high-affinity tail, the Sips distribution decays in the way we have discussed: $\tilde{N}(\Delta G)$ decays as $\mathrm{e}^{-a\Delta G/k_\mathrm{B}T}$, or $N(K)$ as K^{-a-1}. By contrast, a true Gaussian decays much more quickly. Our analysis shows that the tail of the distribution, not the peak, determines the low-concentration limit of the binding isotherm. Hence, the Sips isotherm does not apply to a true Gaussian distribution of ΔG; it applies only to a distribution with the slowly decaying tail.

Our results lead to two main conclusions for experiments. First, if a measured binding isotherm follows the Sips power law of eq 2, then it gives direct information about the high-affinity tail of the affinity distribution $N(K)$. The fitted exponent a implies that $N(K)$ decays as K^{-a-1}. Thus, a is not just an arbitrary index of heterogeneity; it has a specific interpretation in terms of this tail. Second, if a measured binding isotherm follows the linear Langmuir isotherm of eq 1, it *does not imply* that the affinity is homogeneous. It only implies that the affinity distribution is not too heterogeneous; specifically, $N(K)$ decays faster than K^{-2}. Thus, these results provide an important caution for the interpretation of data on binding isotherms for association/dissociation reactions.

DISPLACEMENT REACTIONS

Antibody−antigen reactions do not always have the simple association/dissociation form considered above. In displacement assays, antibodies are preloaded with a fluorescently or radioactively labeled antigen. When the antigen of interest is introduced into the system, it must displace the labeled antigen in order to bind with the antibody. The labeled antigen that is released can then be detected. The displacement reaction can be written as

$$\mathrm{A} + \mathrm{A^*B} \rightleftharpoons \mathrm{A^*} + \mathrm{AB} \tag{15}$$

where A represents the antigen of interest, A* represents the labeled antigen, and B represents the antibody. Analogous displacement processes may take place in surface chemistry if the adsorption centers of a substrate are preloaded with one gas A*, which is displaced by another gas A.

In this section, we investigate the form of the reaction isotherm for a displacement reaction. We do not make any assumptions about the kinetic process of displacement. It does not matter whether A* first dissociates from B and then A binds to B or whether A actively pushes A* off of B. Our analysis applies to any reaction of the form of eq 15.

In our analysis, we suppose that the antigen A is introduced in a low concentration, which can be controlled experimentally. By contrast, the bound labeled antigen A*B is present in a much higher concentration, which is only slightly depleted by the reaction. We also suppose that there is initially no bound antigen A nor free antigen A*; that is, the initial concentrations are $[\mathrm{AB}]_0 = [\mathrm{A^*}]_0 = 0$. In that case, the concentrations [AB] = [A*] always

(10) Goldstein, B. *Biophys. Chem.* **1975**, *3*, 363−367.

remain equal to each other. Both of these assumptions are physically reasonable for an antigen displacement assay, and the analogous assumptions should hold for a surface displacement reaction.

For the displacement reaction 15, the law of mass action states

$$[A^*][AB]/[A][A^*B] = K_R \tag{16}$$

where K_R is the *relative* affinity constant, which represents the affinity of B for A relative to A*. This relative affinity is the ratio $K_R = K/K^*$ of the affinities for A and A*, respectively. For the displacement reaction, we define the reaction coordinate

$$r \equiv \frac{[AB]}{[A^*B] + [AB]} = \frac{[A^*]}{[A^*B] + [AB]} \tag{17}$$

This reaction coordinate represents the fraction of antibodies that are bound to the antigen A, or equivalently, the fraction of antigen A* that has been displaced. By combining the definition (eq 17) with the law of mass action (eq 16), and using $[AB] = [A^*]$, we obtain

$$r = \frac{(K_R[A]/[A^*B])^{1/2}}{1 + (K_R[A]/[A^*B])^{1/2}} \tag{18}$$

This equation is the analogue of the Langmuir isotherm (eq 6) for a displacement reaction. For low concentration [A], it predicts that $r \propto [A]^{1/2}$ instead of $r \propto [A]$. Thus, [AB] increases more quickly as a function of [A] for a displacement reaction than for a simple association/dissociation reaction. This result is reasonable, because the equilibrium value of [AB] depends on the relative rates of the forward and reverse processes. For the displacement reaction, the reverse process is extremely rare, since both [A*] and [AB] are low.

Now, as in the previous section, we suppose that a system contains a heterogeneous mixture of antibodies, with a distribution of affinities K and K^* for antigens A and A*, respectively. Unless K and K^* are perfectly correlated, the antibodies will also have a heterogeneous distribution $N(K_R)$ of the relative affinity K_R. As in the previous section, we suppose that the distribution of relative affinity has the general form illustrated in Figure 1b. The bulk of the distribution may have any arbitrary form, but the high-affinity tail above the threshold affinity K_C decays as a power law: For $K_R \gtrsim K_C$, the distribution becomes

$$N(K_R) = \alpha K_R^{-a-1} \tag{19}$$

To obtain the reaction coordinate r, we integrate over the distribution of relative affinity. This integration gives

$$r = \int_0^\infty dK_R N(K_R) \frac{(K_R[A]/[A^*B])^{1/2}}{1 + (K_R[A]/[A^*B])^{1/2}} \tag{20}$$

To find the leading behavior for low [A], we factor out $([A]/[A^*B])^{1/2}$ and break up the integral into

$$r = \left[\frac{[A]}{[A^*B]}\right]^{1/2}\left[\int_0^{K_C} \frac{dK_R N(K_R) K_R^{1/2}}{1 + (K_R[A]/[A^*B])^{1/2}} + \int_{K_C}^\infty \frac{dK_R \alpha K_R^{-a-1} K_R^{1/2}}{1 + (K_R[A]/[A^*B])^{1/2}}\right] \tag{21}$$

In the limit of low [A], the first integral is a finite constant with respect to [A]. The behavior of the second integral depends on the value of a, but in a slightly different way than in the previous section. Here, if $a > {}^1/_2$, this second integral is also a finite constant with respect to [A]. For low [A], the sum of the integrals then gives

$$r = \overline{K_R^{1/2}}[[A]/[A^*B]]^{1/2} \tag{22}$$

where $\overline{K_R^{1/2}} = \int_0^\infty K_R^{1/2} N(K_R)\, dK_R$. This expression is the low-concentration limit of the modified Langmuir isotherm (eq 18). By contrast, if $a < {}^1/_2$, the second integral diverges as $[A] \to 0$. In that case, the low-concentration limit becomes

$$r = \frac{2\pi\alpha}{\sin 2\pi a}\left[\frac{[A]}{[A^*B]}\right]^a \tag{23}$$

This expression is the analogue of the Sips isotherm (eq 13) for a displacement reaction. In logarithmic form, these results can be summarized as

$$\log r = \begin{cases} {}^1/_2 \log [A] - {}^1/_2 \log [A^*B] + \log \overline{K_R^{1/2}} & \text{for } a > {}^1/_2 \\ a \log [A] - a \log [A^*B] + \log\left[\dfrac{2\pi\alpha}{\sin 2\pi a}\right] & \text{for } 0 < a < {}^1/_2 \end{cases} \tag{24}$$

From these results, we see that displacement reactions are similar in several ways to association/dissociation reactions. The low-concentration limit of the displacement isotherm is dominated by the high-affinity tail of the distribution of relative affinity. If the system is homogeneous, or if it is only moderately heterogeneous, the displacement isotherm takes the modified Langmuir form. By contrast, if the affinity distribution is very heterogeneous, the displacement isotherm takes the Sips form. Those results are analogous to the results for association/dissociation reactions seen earlier. However, there are two essential differences between displacement and association/dissociation reactions. First, the modified Langmuir isotherm for a displacement reaction predicts the square-root law $[AB] \propto [A]^{1/2}$, in contrast to the linear Langmuir isotherm $[AB] \propto [A]$ for an association/dissociation reaction. Second, the threshold for the crossover from the modified Langmuir to the Sips isotherm is $a = {}^1/_2$ for a displacement reaction, in contrast with the threshold of $a = 1$ for an association/dissociation reaction. Thus, a displacement reaction is *less sensitive* to heterogeneity than an association/dissociation reaction. It takes a greater level of heterogeneity (a more slowly decaying tail of the affinity distribution) to change the isotherm to the Sips form in a displacement reaction than in an association/dissociation reaction.

DISCUSSION

The results of this paper have broad implications for experiments that assess heterogeneity in antibody−antigen binding,

surface adsorption, or other reactions. First, we have shown that isotherms for association/dissociation reactions provide only a limited amount of information about heterogeneity. If the isotherm follows the Sips power law $[AB] \propto [A]^a$, with $0 < a < 1$, then one can extract information about the tail of the heterogeneous distribution: For high affinities, $N(K)$ decays as K^{-a-1}, or equivalently, $\tilde{N}(\Delta G)$ decays as $e^{-a\Delta G/k_B T}$. However, one cannot extract any information about the shape or width of the peak in the distribution. Furthermore, if the isotherm follows the Langmuir form $[AB] \propto [A]$, one cannot infer that the affinity is homogeneous. The affinity could be homogeneous or it could be moderately heterogeneous, with $N(K)$ decaying faster than K^{-2}.

In addition, we have shown that isotherms for displacement reactions provide somewhat less information about heterogeneity than isotherms for association/dissociation reactions. If the displacement isotherm follows the Sips power law $[AB] \propto [A]^a$, with $0 < a < {}^1/_2$, then one can infer that the tail of $N(K_R)$ decays as K_R^{-a-1}. Again, one cannot extract any information about the shape or width of the peak in $N(K_R)$. By contrast, if the isotherm follows the modified Langmuir form $[AB] \propto [A]^{1/2}$, one cannot infer that the affinity is homogeneous. It could be moderately heterogeneous, with $N(K_R)$ decaying faster than $K_R^{-3/2}$. Those results are all analogous to results for association/displacement reactions, but the threshold for the crossover between the Sips and (modified) Langmuir forms is $a = {}^1/_2$ rather than $a = 1$. As a result, displacement reactions are less sensitive to heterogeneity than association/dissociation reactions. A distribution must be more heterogeneous to change the exponent in a displacement isotherm than to change the exponent in an association/dissociation isotherm.

In the accompanying paper, Rabbany et al.[11] present experimental data for an antibody−antigen displacement reaction. In those experiments, a flow immunosensor was used to measure the displacement isotherm for a system of monoclonal antibodies sensitive to trinitrotoluene (TNT). In logarithmic form, the experimental results show that $\log [AB] = a \log [A] + \text{const}$, with $a = 0.51 \pm 0.11$. Prior to this theoretical analysis for displacement reactions, one might have assumed that this value of a is the index of heterogeneity in the Sips isotherm. Because this value is much less than 1, it would have implied that the antibody system is quite heterogeneous. That result would indeed have been paradoxical, because a system of monoclonal antibodies should be quite homogeneous. However, our analysis shows that the measured displacement isotherm is precisely the modified Langmuir form, which is consistent with a homogeneous or only moderately heterogeneous distribution of relative affinities. One would have to use a much more heterogeneous distribution, perhaps in a system of polyclonal antibodies, to see the Sips isotherm in a flow immunosensor.

In conclusion, we have analyzed the effects of a heterogeneous distribution of affinities, using a very general model for the shape of affinity distribution. Our analysis shows that heterogeneity substantially changes the form of reaction isotherms if the tail of the distribution decays sufficiently slowly. However, heterogeneity does not change the form of reaction isotherms if that tail decays rapidly. Thus, our analysis provides a very general caution for experiments that use reaction isotherms to assess heterogeneity.

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(11) Rabbany, S. Y.; Piervencenzi, R.; Judd, L.; Kusterbeck, A. W.; Bredehorst, R.; Hakansson, K.; Ligler, F. S. *Anal. Chem.* **1997**, *69*, 175−182.