Precipitation at Equivalence and Equilibrium: A Method for the Determination of Equilibrium Constants of Reaction between Multideterminant Antigen and Specific Polyclonal Antibodies

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A theoretical approach for the determination of the equilibrium constant, K_a, of the reaction between a multideterminant antigen (Ag) and specific polyclonal antibodies (Ab) forming the insoluble Ab/Ag immune complex, is derived. The constant can be expressed as a function of the two accessible experimental parameters, the precipitating concentration of the antigen and the Ab/Ag molar ratio. For this purpose Ab/Ag immune complex must be prepared at equivalence, and equilibrium between precipitated and soluble species must be reached. The proposed method is experimentally tested on the system human serum albumin (HSA) and polyclonal rabbit antibodies. The Ab/Ag precipitates are prepared by the direct mixing of biological fluids in which immunoreacting components naturally occur. Previous separation, purification, or labeling of immunoreacting components are not required. The conditions for the precipitation of Ab/Ag complexes at equivalence, the stoichiometric composition or the average number of Ab molecules bound to one Ag molecule, and the solubility of the immunoprecipitating components are determined by a rectangular two-dimensional double immunodiffusion. Since the solubility determined under the conditions of a double immunodiffusion is a result of the interaction of the global diffusion of the precipitating components and particle growth kinetics, it mostly refers to the dynamic conditions. To find the solubility under equilibrium conditions, it is sufficient to determine the minimal factor by which the solutions of both immunoprecipitating components should be diluted so that no precipitate is formed upon their mixing at equivalence. The dilution factor is determined by a measurement of the laser light scattering of the immunoprecipitating systems prepared with serially diluted Ag and Ab solutions.

INTRODUCTION

Affinity, specificity, and concentration of an antibody (Ab) determine its usefulness for analytical, diagnostic, and therapeutic purposes and are also implicated as important factors in the immune response. Antibody affinity is defined as the attractive force between an antigenic determinant (epitope) and the antibody combining site (paratope). Accordingly to its definition, the affinity can be measured only when the antigen (Ag) is a simple, well-defined substance such as a hapten. The determination of the affinity of antibodies directed against a protein in this sense is impossible because of the multiplicity and heterogeneity of antigenic determinants. To describe quantitative differences of the interactions between a multideterminant Ag and polyclonal Abs, despite a lack of a precise thermodynamic and immunochemical meaning, the term avidity was introduced. Avidity is the goodness of fit between more than one epitope of the antigen and more than one site of the antibody. The rates of dissociation, the solubilities of Ab/Ag complexes precipitated at the optimum proportions, the deviations from the linearity of the curve representing reciprocal concentrations of a bound antigen vs reciprocal concentrations of a free antigen, antigen binding capacities, immunochemical titers, indices of avidities, as well as the data obtained by measurements of 50% binding of antibodies by an antigen are related to the avidity.[1−3] However, the avidities are often expressed through the association constants, K_a, which represent the equilibrium of association, k_a, and dissociation, k_d, rates of Ab/Ag complexes. Considering the difficulties in reaching equilibrium due to heterogeneous binding, the application of the solid phase affinity methods for the determination of K_a should be taken with precautions. Despite the simplicity of these methods, such as an ELISA, various surface effects can cause errors in estimates of either liquid or solid phase affinities and can influence the ranking of affinities.[2,4,5]

In this paper we propose a new approach to the determination of the equilibrium constants of the reactions between multideterminant Ags and specific polyclonal Abs. The method is based solely on the determinations of the concentration of soluble Ag in equilibrium with insoluble Ab/Ag immune complex prepared at equivalence.

THEORETICAL CONSIDERATIONS

Equilibrium Constants of Polyclonal Antibodies Raised against a Multideterminant Antigen. An immune complex of the average composition AB_n is formed when one molecule of multideterminant antigen A reacts with n molecules of the specific polyclonal antibodies B:

$$A + nB \leftrightarrow AB_n$$

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The average composition means that n should not necessary be an integer number. In the case that n is greater than unity, a portion of the immune complex AB_n may be precipitated. In this case, the equilibrium is reached when fully reversible reaction is established between the precipitated complex AB_{np} (solid phase) and the species remaining in the solution (solute phase):

$$AB_{np} \leftrightarrow AB_{ns} + A_s + nB_s$$

The solute phase consists of the free components A_s and B_s and components $A_{s,b}$ and $B_{s,b}$ bound in the soluble AB_{ns} complex. The reversible reaction between soluble immune complex and free species in solute phase also exists under equilibrium conditions:

$$AB_{ns} \leftrightarrow A_s + nB_s \tag{1}$$

In the immunoprecipitating system prepared under equivalence conditions, the ratio of molar concentrations of B and A components, n, is

$$n = cB/cA \tag{2}$$

and is the same in the precipitated and soluble immune complexes and corresponds to the ratio of free B and A species in the solute phase.

According to eq 1, the equilibrium constant of the primary Ab/Ag reaction in a system prepared at equivalence and equilibrium is

$$K'_a = cA_{s,b}/(cA_s\, cB_s^{\,n}) \tag{3}$$

By substitution of the cB by cA from eq 2, the equilibrium constant equation becomes

$$K'_a = cA_{s,b}/(n^n\, cA_s^{\,n+1}) \tag{4}$$

K'_a reflects the avidity of the antibodies, irrespective of the number of antibody valences, i.e., binding sites, involved in the reaction. In the case that the multideterminant antigen bears distinct antigenic determinants, each of them is able to react only with one binding site on the molecule of the specific antibody. Since the antibody molecules possess more than one binding site, the valency of antibodies should be taken into account in order to calculate the equilibrium constant. For instance, the equilibrium constant of the reaction involving bivalent antibodies belonging to IgG classes, reads as

$$\ln K_a = \ln K'_a/2n \tag{5}$$

Equivalence and Equilibrium Conditions. In the case that the concentrations of the antigen and antibody solutions are unknown, the equivalence conditions can be determined by a rectangular two-dimensional double-diffusion technique called the "two-cross" immunodiffusion. A detailed description of the two-cross experimental setup which enables an adequate solution of Fick's second law of diffusion applied to the immunoprecipitation in gels is described elsewhere.[6,7] Briefly, a "cross" consists of four troughs cut at a right angle in a gel plate. The half-width of the trough is denoted by h. The troughs of each cross are filled in alternate order with antigen solution (component A) and immune serum (component B). In the second cross, the solutions of both precipitating components are diluted by the same factor, d. The distances between peaks of the precipitin lines in the direction of the diffusion of antigen, x, and antibodies, y, are measured in both crosses at a same time, t. The volume ratio of the solutions of the precipitating components required to ensure equivalence conditions during the preparation and precipitation of immune complexes[8] is given by

$$vB/vA = ad^b \tag{6}$$

where

$$a = [(x_1^{\,2} - x_2^{\,2})/(y_1^{\,2} - y_2^{\,2})]^{1/2}$$

$$b = (x_2^{\,2}y_1^{\,2} - x_1^{\,2}y_2^{\,2})/[(x_1^{\,2} - x_2^{\,2})(y_1^{\,2} - y_2^{\,2})]$$

The subscript 1 and 2 refer to the parameters measured in the first and the second cross, respectively.

So far, the two-cross immunodiffusion technique enables the direct determination of the reciprocal precipitating titers (eqs 7 and 8) and the diffusion coefficients (eqs 9 and 10) of the reacting molecules. Precipitating titers PT are defined as the ratio of the equivalent molar concentrations of the substance at the origin of diffusion, c_0, and at the point of the onset of precipitation, c_{pr},

$$1/PTA = (W/x_1)X\, e^{-X} = cA_{pr}/cA_0 \tag{7}$$

$$1/PTB = (W/y_1)Y\, e^{-Y} = cB_{pr}/cB_0 \tag{8}$$

where

$$W = h(2/\pi)^{1/2}$$

$$X = x_1[2 \ln d/(x_1^{\,2} - x_2^{\,2})]^{1/2}$$

$$Y = y_1[2 \ln d/(y_1^{\,2} - y_2^{\,2})]^{1/2}$$

The precipitating concentrations, c_{pr}, can be calculated from both known precipitating titers and known initial concentrations of the solution of the precipitating components. In the case that the initial concentrations of the antibody and antigen solutions are unknown, they can be determined directly in crude biological fluids in a manner described in detail elsewhere.[8,9]

The diffusion coefficients are obtained from the relations:

$$D_A = (1/t)[(x_1^{\,2} - x_2^{\,2})/(4 \ln d) - h^2/6] \tag{9}$$

$$D_B = (1/t)[(y_1^{\,2} - y_2^{\,2})/(4 \ln d) - h^2/6] \tag{10}$$

From the data obtained for the diffusion coefficients, the approximate values of the molecular masses of the Ag and Ab molecule can be calculated using a simple relation:

$$M = M_{IgG}\,(D_{IgG}/D)^3 \tag{11}$$

$M_{IgG} = 150\,000$ Da and $D_{IgG} = 4.1 \times 10^{-7}$ cm^2/s are the molecular mass and the diffusion coefficient of human IgG, respectively. D is the diffusion coefficient of the Ag or Ab molecule referring to the free diffusion in distilled water at 20 °C.

From the conservation of mass, the precipitating concentrations referring to the equilibrium conditions correspond to the sum of the concentration of free reactant and the concentration of reactant bound in a soluble immune complex:

$$cA_{pr} = cA_{s,b} + cA_s \quad (12)$$

$$cB_{pr} = cB_{s,b} + cB_s \quad (13)$$

According to the equivalence rule, the precipitation under conditions of a double diffusion starts at the equivalent molar concentrations of both precipitating components,[7,10−14] as indicated by eq 2:

$$n = cB_{pr}/cA_{pr} \quad (14)$$

This means that the equilibrium constant equation (eq 4) can be solved using c_{pr} values from eq 7 or 8. The critical precipitating concentrations in eqs 7 and 8 represent the solubility of an immunoprecipitating component under dynamic conditions, as a result of the interaction of the global diffusion of the precipitating components and particle growth kinetics.[10] Thus, the solubility, c_{pr}, has a kinetic and not a thermodynamic significance. In the case when enough time is allowed for the precipitation in the solutions to reach the equilibrium, the solubility referring to the thermodynamic conditions could be found. If not, it is sufficient to determine the minimal factor, m_0, by which both solutions of A and B components should be diluted so that no precipitate is formed upon their mixing at equivalence. The concentrations, c_{pr}/m_0, referring to the equilibrium conditions are denoted as c_{pr}*.

To solve eq 4 using cA_{pr}* and/or cB_{pr}*, the concentrations of free components and components bound in an immune complex in the solute phase (eqs 12 and 13) should be known. The solution is possible by introducing the ratio, r, of the concentrations of both bound and free antigen in the solute phase:

$$r = cA_{s,b}*/cA_s*$$

The equilibrium concentrations cA_s* and $cA_{s,b}$ *, according to the definition for cA_s and $cA_{s,b}$ given by eq 12, can be now expressed as follows:

$$cA_s* = cA_{pr}*/(r+1) \quad (15)$$

$$cA_{s,b}* = cA_{pr}*r/(r+1) \quad (16)$$

The equilibrium constant equation (eq 4) in terms of cA_{pr}*, r, and n, reads as

$$K'_a = (1/cA_{pr}*)^n r[(r+1)/r]^n \quad (17)$$

Equation 17 consists of two factors: the factor $(1/cA_{pr}*)^n$ and a factor F which is a function of r and n:

$$F = r[(r+1)/n]^n \quad (18)$$

The molar concentrations of both bound and free antigen in the solute phase are extremely low, and their experimental determination as well as the determination of their ratio, r, is difficult. By varying the values of n in eq 18, the F function reaches a maximum for a constant r value (Figure 1). The maximum of the F function corresponds to the maximal stability of an immunoprecipitating system prepared at equivalence under given experimental conditions (pH, temperature, ionic strength, etc.), and now, the K'_a equation reads as

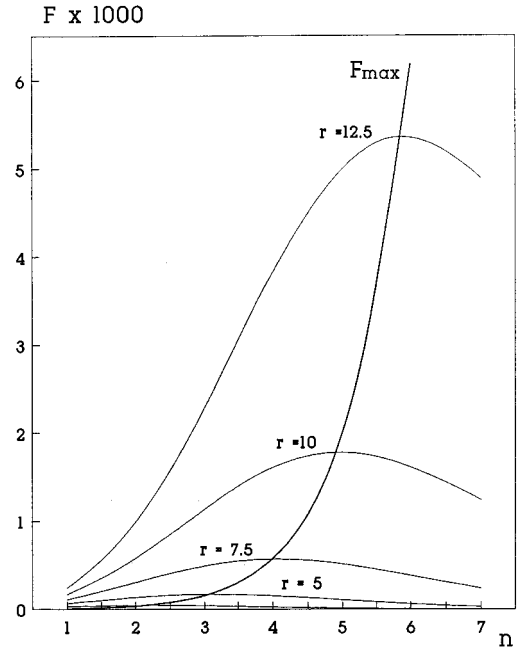

Figure 1. Illustration of the function $F = r[(r+1)/n]n$ vs n for some values of r.

$$K'_a = (1/cA_{pr}*)^n F_{max} \quad (19)$$

F_{max} is solely a function of n. To find F_{max}, the derivative of F should be zero:

$$dF/dn = (r/n^2)[(r+1)^n \ln(r+1) - (r+1)^n(\ln n + 1)] = 0$$

This means that

$$\ln(r+1) = \ln n + 1 = \ln(n\,e)$$

i.e.,

$$r = n\,e - 1 \quad (20)$$

Introducing eq 20 into eq 18

$$F_{max} = (n\,e - 1)e^n \quad (21)$$

where e is the basis of the natural logarithms.

The solution of the equilibrium constant eqs 4 and/or 17 in terms of cA_{pr}* and n is:

$$K'_a = (e/cA_{pr}*)^n(n\,e - 1) \quad (22)$$

MATERIALS AND METHODS

Immunogen and Antigen. Immunochemically pure human serum albumin (HSA), pI 4.7, MW 65 kDa, was prepared under nondenaturating conditions and contained 97.6% of monomer and 2.4% of dimer (Calbiochem-Behring Corp., La Jolla, CA). The same HSA was used for the immunization of rabbits (immunogen) and as the antigen in

the precipitation experiments. The concentration of antigen solutions amounted to $cA_0 = 0.500$ g HSA/L.

Animals, Immunization, and Immune Sera. Three randomly chosen male New Zeland rabbits were immunized successively in two-week intervals with the HSA. Each dose of the immunogen contained 0.75 mg of the HSA in a volume of 1 mL of complete (CFA) or incomplete Freund's adjuvant (IFA) or saline solution. For the primary immunization, the immunogen was emulsified in the CFA and administered i.d. in the region of the peritoneal cavity. For the first and the second booster doses, the HSA was emulsified in the IFA and administered i.d. in the region of the peritoneal cavity. For the third booster dose, a solution of HSA in saline was applied i.v. in ear veins.

Two weeks after receiving the last booster dose, the rabbits were bled and the blood samples collected separately. The immune sera were decomplemented at 56 °C for 30 min. The antibody concentrations of the immune sera were previously determined by a microgravimetric method.[8]

The Two-Cross Immunodiffusion Experiments. For the two-cross immunodiffusion experiments 1% w/v agarose gel was prepared using agarose L (Behring Institute, W. Germany). Phosphate-buffered saline (PBS), pH 5.0, 5.5, and 7.0, contained 0.05 M KH_2PO_4, 0.10 M NaCl, 0.1% w/v NaN_3, and a variable amount of NaOH. The borate-buffered saline (BBS) contained 0.05 M H_3BO_3, 0.1 M NaCl, and 0.1% w/v NaN_3, and NaOH was added in order to reach pH 8.6. These buffer solutions were used to dilute the precipitating components and to equilibrate the agarose in which the two-cross immunodiffusion experiments were performed. The immunodiffusion experiments were carried out at 20 and 40 °C. For all technical details concerning the two-cross immunodiffusion experimental procedure and the evaluation of the results, refer to Pokrić and Pučar[6] or Živković et al.[7] for somewhat less-detailed descriptions.

The Determination of the Solubility of Immune Complexes. The solubility of the immune complexes was determined by consecutive dilutions of the solutions of the precipitating components until reaching the dilution, m_0, at which no precipitate is formed.[8] The starting precipitating system was prepared at equivalence by mixing 20 μL of the immune serum and a volume of antigen solution that was calculated by eq 6. For the dilutions, the total volume of the precipitating system was maintained constant, but the volume parts of the solutions of the precipitating components were subsequently reduced and simultaneously the volumes of the buffer solution increased. Relative laser light scattering (%RLLS) measurements were performed using a Hyland laser nephelometer PDQTM (Travenol Laboratories, Costa Mesa, CA). For each dilution, the measurement was carried out until the maximum values of scattered light, $(\%RLLS)_{max}$, were reached. The experiments were performed at 20 and 40 °C.

RESULTS

Figure 1 illustrates the changes of the factor F (eq 18) vs n for certain values of r. The molar Ab/Ag ratio, n, refers to the immune complex prepared at equivalence. The ratio of the molar concentrations of bound Ag over free Ag, $r = cA_{s,b}^*/cA_s^*$, is related to the equilibrium reached in the solute phase. Figure 1 shows that for each of the chosen r values ranging between 5 and 12.5 and with varying n values from 1 to 7, the factor F reaches a maximum. The n values must lay in a limited range. The values of $n < 2$ will seldom give precipitates at equivalence. The values of $n > 5$ will exceptionally occur, and then the maxima of the F function vs n (Figure 1) will have a very steep shape. The latter case would drastically increase the equilibrium constant (eq 19). For n values between 1.5 and 5.5, and according to eq 21, the values of the F_{max} will lay between 13.79 and 3412. From eq 18 proceedes that in this case the molar ratios, r, of associated antigen, $cA_{s,b}$, and free antigen, cA_s, in the solute phase will predominantly lay between 3.07 and 13.95.

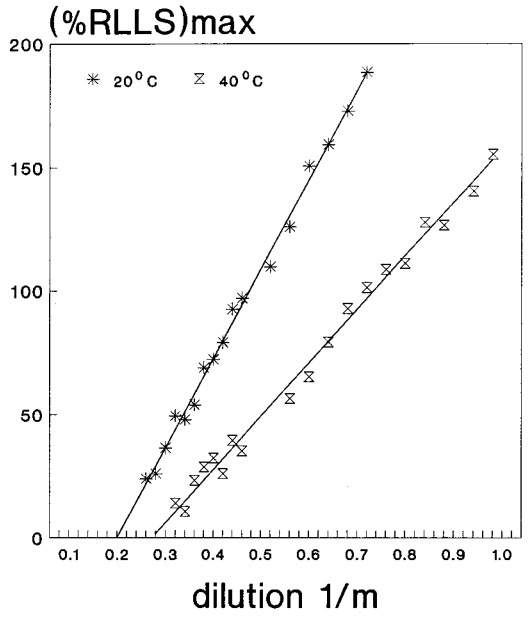

Figure 2. Determination of the minimal dilution factor, m_0, necessary to obtain a system free of precipitate. The maximal laser light scatter $(\%RLLS)_{max}$ is presented as the function of the serial dilutions, m, of a given HSA−antiHSA system prepared at the equivalence at 20 and at 40 °C. The immunoprecipitation system is prepared at pH 7.0 by using the immune serum of rabbit 2. Regression coefficient is 0.991 and 0.993 at 20 and 40 °C, respectively.

The diffusion coefficients of antigen and antibody, $D_A = 6.1 \times 10^{-7}$ cm^2/s and $D_B = 4.1 \times 10^{-7}$ cm^2/s, determined by the immunodiffusion method (eqs 9 and 10), correspond to the HSA and IgG class rabbit antibodies having molecular masses $M_A = 65\,000$ Da and $M_B = 160\,000$ Da (eq 11), respectively.

An example of the determination of the dilution factor, m_0, at which no detectable precipitate is formed is presented in Figure 2. The quantity of the precipitate formed was determined by the laser light scattering measurements. A strict linearity, $r > 0.9$, between the intensity of scattered light, $(\%RLSS)_{max}$, and the dilution, $1/m$, was obtained in all examined systems. Thus, it is not necessary to find experimentally the dilution at which precipitation occurs anymore. The extrapolation of the straight line $(\%RLSS)_{max}$ vs $1/m$ to %RLLS = 0 gives the required dilution $1/m_0$.

The precipitating titers of both reactants determined by the two-cross immunodiffusion (eqs 7 and 8) and the dilution factors required for the corrections to equilibrium conditions are presented in Table 1. The values obtained for the dilution factor, $m > 1$, proved that the precipitating titers determined

Table 1. Precipitating Titers of the Antigen (PTA) and Antibody (PTB) Solutions and the Minimal Dilution Factors (m_0) in antiHSA/HSA Systems[a]

immune serum	pH	20 °C PTA	20 °C PTB	20 °C m_0	40 °C PTA	40 °C PTB	40 °C m_0
1	5.0	356	265	3.95	549	400	1.66
	5.5	509	262	4.90	565	293	3.22
	7.0	509	348	2.71	562	384	1.79
	8.6	659	412	3.48	735	434	2.37
2	5.0	339	377	5.99	387	448	4.93
	5.5	470	373	7.75	551	448	6.37
	7.0	487	470	5.15	551	554	3.72
	8.6	682	604	5.88	718	638	4.86
3	5.0	270	189	3.72	462	321	1.94
	5.5	382	189	4.76	554	271	3.21
	7.4	382	250	3.02	547	349	1.93
	8.6	508	280	3.10	761	407	1.52

[a] The precipitating titers and dilution factors are dimensionless quantities.

Table 2. Precipitating Concentrations (c_{pr}) and the Ab/Ag Molar Ratio (n) in the antiHSA/HSA System Prepared at Equivalence

immune serum	pH	20 °C $c_{pr}/10^3$ [a] A	20 °C $c_{pr}/10^3$ [a] B	20 °C n[b]	40 °C $c_{pr}/10^3$ [a] A	40 °C $c_{pr}/10^3$ [a] B	40 °C n[b]	n[b]
1	5.0	1.4045	6.6868	2.06	0.9108	4.4300	2.11	2.085
	5.5	0.9823	6.7633	2.98	0.8850	6.0477	2.96	2.970
	7.0	0.9823	5.0919	2.25	0.8897	4.6145	2. 25	2.250
	8.6	0.7194	4.3009	2.59	0.6803	4.0829	2.60	2.595
2	5.0	1.4749	7.1618	2.10	1.2920	6.0268	2.02	2.060
	5.5	1.0638	7.2386	2.95	0.9074	6.0268	2.88	2.915
	7.0	1.0267	5.7447	2.43	0.9074	4.8736	2.33	2.380
	8.6	0.7331	4.4702	2.64	0.6964	4.2320	2.63	2.635
3	5.0	1.8519	7.1058	1.67	1.0823	4.1838	1.68	1.675
	7.0	1.3089	5.3720	1.78	0.9141	3.8481	1.82	1.800
	8.6	0.9843	4.7964	2.11	0.6570	3.2998	2.18	2.145

[a] The precipitating concentrations, expressed as grams per liter, are calculated (eqs 7 and 8) taking into account that the concentration of HSA solutions (A) amounted to $cA_0 = 0.500$ g/L and the concentrations of antiHSA in rabbit sera (B) amounted to $cB_0 = 1.772$ g/L (rabbit 1), $cB_0 = 2.700$ g/L, (rabbit 2), and $cB_0 = 1.343$ g/L (rabbit 3).[8] [b] n is calculated (eq 14) taking into account that the molecular masses of antigen and antibodies amount to 65 000 and 16 0000 Da, respectively.

under conditions of the double diffusion do not refer to equilibrium conditions. Table 1 and Figure 2 show that m_0 is always smaller at 40 °C than at 20 °C. This suggests that the precipitation under conditions of diffusion at higher temperatures occurs under conditions closer to that of equilibrium.

The precipitating concentrations, c_{pr}, of antigen and antibodies at which the precipitation starts under conditions of double diffusion (eqs 7 and 8) are presented in Table 2. Taking into account antibody and antigen molecular masses, the molar Ab/Ag ratio, n, required for the formation of the immune complex at equivalence was calculated (eq 14). The stoichiometric composition of an Ab/Ag complex prepared under identical experimental conditions but at two different temperatures is constant (Table 2). The small differences between n at 20 °C and at 40 °C arise from the errors in experimental determinations of the precipitating concentrations, cA_{pr} and cB_{pr} (eqs 7 and 8).

The mean values of n are used in order to calculate the equilibrium constants K'_a (eqs 22). To calculate K'_a, the precipiting concentrations of antigen solutions obtained in grams per liter (Table 2) must also be expressed in moles per liter and corrected to equilibrium conditions, cA_{pr}*, by using dilution factors, m_0 (Table 1). Assuming that IgG class Abs are bivalent, the equilibrium constants, K_a, are calculated according to eq 5 and presented in Table 3. The precipitating titers of the antigen solution corrected to equilibrium conditions, PTA*, are also presented in Table 3. Although in our experiments the same antigen solution of a constant concentration, cA_0, was used, different PTA* values were obtained under different experimental conditions. However, the data presented in Table 3 show that K_a and PTA* values are well-related. Thus, the PTA* data could be used for the rough ranking of the avidities of different immune sera for the same multideterminant antigen.

Table 3. Precipitating Titers of Antigen Solution (PTA*) Referring to the Equilibrium Conditions and the Equilibrium Constants of AntiHSA/HSA Reactions (K_a)

immune serum	pH	20 °C PTA* [a]	20 °C $K_\alpha/10^{-4}$ [b]	40 °C PTA* [a]	40 °C $K_\alpha/10^{-4}$ [b]
1	5.0	1406	3.23	911	2.60
2	5.0	2031	3.89	1908	3.76
3	5.0	1004	2.75	869	2.60
1	5.5	2494	4.13	1819	3.53
2	5.5	3643	5.00	3510	4.19
3	5.5	1818	3.62	1778	3.58
1	7.0	1379	3.17	1006	2.71
2	7.0	2508	4.25	2066	3.85
3	7.0	1154	2.95	1056	2.82
1	8.6	2419	4.14	1742	3.51
2	8.6	4010	5.13	3490	4.95
3	8.6	1575	3.41	1157	2.92

[a] The precipitating titers are dimensionless quantities. [b] The equilibrium constants are expressed in liters per mole and calculated according to eqs 5 and 22. For this purpose the precipitating concentrations of antigen solutions in grams per liter (Table 2) are transformed to equilibrium conditions by using the dilution factor m_0 (Table 1) and are expressed in moles per liter, taking into account that the molecular mass of antigen is 65 000 Da. The average of the n values determined at 20 and 40 °C (Table 2) is introduced into eqs 5 and 22.

Lower K_a values (Table 3) at higher rather than at lower temperatures suggest that antiHSA/HSA binding is an exothermic process.

DISCUSSION

The method, proposed for the determination of the equilibrium constants of the reaction of the mutideterminant antigen and specific polyclonal antibodies, requires only the knoweledge of two accessible experimental parameters: the concentration at which the antigen starts to precipitate under equilibrium conditions and the molar Ab/Ag ratio in the immune complex prepared at equivalence. The immune complexes can be prepared by the direct mixing of biological fluids in which immunoreacting components naturally occur. This offers a great advantage of dealing with unmodified molecules, since the separation, purification, and labeling of either the antigen or the antibody, which might modify the binding properties,[15] is not required.

Different values of precipitating titers of the antigen solution corrected to equilibrium conditions, PTA* (Table 3), obtained for the same Ab/Ag system at different pH values as well as under the identical experimental condition at two different temperatures (Table 1) proved that the

solubility, cA_{pr} (eq 8), of a HSA−anti HSA system is dependent on both pH and temperature. The changes of the stoichiometry of an antiHSA−HSA pair at various pH values (Table 2), previously observed in the experiments with a number of Ab/Ag complexes,[8,16,17] are caused by the changes of the charge of Ab and Ag molecules with ambient pH.[18,19] The dependence of both the solubilty and stoichiometry of the Ab/Ag system on the experimental conditions explains the variation of equilibrium constant values of a given antiHSA−HSA pair at various experimental conditions (Table 3) since K_a is directly related to the solubility, cA_{pr}, and Ab/Ag ratio, n, (eqs 5 and 22). This finding agrees with the literature data that the value of the equilibrium constant of an Ab/Ag system is affected by the conditions under which the determination was carried out.[1,18]

Part of the difficulty in the determination of the equilibrium constants of reaction between multideterminant antigen and polyclonal antibodies lies in the fact that K_a values are often dependent upon the absolute amounts of antigen and antibodies, the dilution and/or the volume of the immunoreacting system, as well as upon the ratio of Ab/Ag concentrations.[20−25] So far, the state of equilibrium is disturbed and the dissociation rate is greatly increased when one of the precipitating components is present in a great excess.[26] In our experiments the Ab/Ag concentration ratio is determined in advance by preparing the precipitating system at equivalence, while the K_a determined at equilibrium is invariable to the total concentrations of antigen or antibodies in biological fluids.

The determination of the equilibrium constant K_a in our experiments was possible from the data obtained by the two-dimensional double immunodiffusion concerning the preparation of an Ab/Ag system at equivalence, the precipitating titer of antigen solution, PTA, and diffusion coefficients, D, and/or molecular masses, M, of immunoreacting molecules. The concentration of antigen solution, cA_0, should be known or determined in advance in order to calculate the critical precipitating concentration of antigen, cA_{pr} (eq 7), required for the determination of K'_a (eq 22). The determination of the precipitating titer (PT) by the two-cross immunodiffusion does not require the use of the standards or the knowledge of the concentrations of the solutions of the precipitating components. For a constant concentration of the antigen solution, cA_0, the precipitating titers referring to equilibrium conditions, PTA*, depend solely on the critical precipitating concentrations, cA_{pr}* (eq 7). So far, according to the theory, the different concentrations of identical antibodies obtained by dilutions of an immune serum would not influence the PTA* and/or cA_{pr}* values.[8] The comparison of PTA* and K_a values (Table 3) shows that they are well-related. Thus, under the conditions when the concentrations and molecular masses of antigens and antibodies are unknown or difficult to determine, the PTA* could be used for a rough ranking of relative affinities of different immune sera against the same antigen.

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