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AFFINITY CONSTANTS IN IMMUNOADSORPTIVE CHROMATOGRAPHY: THEIR SIMPLE DETERMINATION AND APPLICATION TO THE SELECTION OF ELUENTS IN AND OPTIMIZATION OF THE ELUTION PROCESS

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SUMMARY

A simple method is described for the estimation of affinity constants of immobilised antibodies by measuring the ratio of bound antigen to immobilised antibody by the Mancini immunodiffusion technique, and representing the data in the form of a Langmuir adsorption isotherm plot of the reciprocal of the ratio of the bound antigen to immobilised antibody as ordinate against the reciprocal of the unbound antigen as abscissa. The slope of the resulting straight line is proportional to the equilibrium constant and the intercept on the ordinate is the reciprocal of the antibody valency. Sheep anti-human IgG immobilised on cyanogen bromide-activated Sepharose 4B® was employed in the measurement of the affinity constant for the soluble affinity purified human IgG in several potential eluents. The qualitative and the quantitative effect of four potential eluents on human IgG antigen and sheep anti-human IgG antibody incubated in the four eluents for 24 h were also assessed by the Mancini technique. These results emphasise the importance of a knowledge of the affinity constant combined with quantitative data and the effect of eluents and buffers on antibodies and antigens in immunoadsorption and other immunochemical procedures.

INTRODUCTION

High affinity constants are characteristic of antigen–antibody interactions. Consequently, the isolation of antigens or antibodies by immunoadsorbents often requires the use of drastic desorbing agents such as glycine–HCl (0.1 *M*, pH 2.5)[1], ammonia solution (0.5 *M*, pH 11.5)[2] and chaotropic solutions of ammonium thiocyanate (3 *M*), trichloroacetate and perchlorate[3]. Although cyanogen bromide-activated Sepharose 4B is the most widely used water-insoluble carbohydrate matrix for the synthesis of immunoadsorbents[4], the matrix has been observed to be unstable in hot water, alkali and non-aqueous solvents[5]. Furthermore, the ligand–matrix bond has been shown to be unstable in the presence of certain amine buffers[6–8]. Despite the widespread use of immunoadsorption techniques for the purification of antigen and

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antibodies, data on the effect of some common eluents on these proteins is very scant in the literature.

Bartels *et al.*[9] have described the effect of three buffers, Tris–HCl (0.075 *M*, pH 8.6), barbital (0.075 *M*, pH 8.6) and borate (0.075 *M*, pH 8.6), on the binding of thyroxine (T_4) to the serum proteins thyroxine-binding globulin (TBG), pre-albumin (TBPA) and human serum albumin (TBA). Their results indicated that barbital buffer served to inhibit the interaction of TBA and TBPA with T_4 and was therefore the buffer of choice since it produced the highest retention of $[^{125}I]T_4$ on a Sephadex column. These results accorded with the paper electrophoresis studies of Tata *et al.*[10].

In our immunoadsorption studies, we have investigated the effect of four potential eluents on human immunoglobulin G (IgG) antigen and sheep anti-human IgG antibody and found that glycine–HCl (0.2 *M*, pH 2.2) had the most severe effect on the avidity of the antigen and antibody, reflected in a marked reduction in the Mancini precipitin ring diameter with time of incubation. A simple method is described for determining the affinity constant of an immobilised antibody for the soluble complementary antigen in different potential eluents. Such a constant provides the fundamental index that will best allow prediction of separation efficiency in the application of immunoadsorbents.

The Scatchard and Langmuir forms of the affinity equations are widely used in immunochemistry to evaluate the association constant commensurate with homogenous binding. However, multifunctional receptors give rise to cooperational effects in the binding process which demand additional parameters to fully characterise the reaction. Both Scatchard[11] and Langmuir[12] plots deviate from linearity in such cases and would have a positive and negative derivative. A Scatchard plot obtained for a receptor population which is heterogenous with respect to its binding force energy for ligand, but non-cooperative is indistinguishable for a plot obtained with a negatively cooperative system[13]. In such cases the intrinsic affinity constant may only be an average of high and low binding antibodies which require, respectively, low and high free component contributions to satisfy the equilibrium condition.

Karush and Soneberg[14], Nisonoff and Pressman[15,16], Bowman and Aldjem[17] and Thakur and Delisi[13] have proposed several modifications to the equilibrium equations to extract from equilibrium binding data free energy distribution functions. These modifications are in general mathematically involved and practically tedious. In contrast, the interaction of immobilised heterogenous antibodies with affinity purified antigen provides a good fit to the Langmuir form of the affinity equation.

MATERIALS AND METHODS

CNBr-activated Sepharose 4B was obtained from Pharmacia (Uppsala, Sweden). Human IgG (Batch D152, 10.5 mg ml^{-1}) and sheep anti-human IgG (batch Z511G, 24.7 mg ml^{-1}) and NIRDL (normal human serum code No. BR 99) serum standard were obtained as solutions from Seward Labs. (London, Great Britain). Agarose for immunochemical studies was purchased from Fisons (Loughborough, Great Britain). All reagents for the preparation of buffers and salt solutions were AnalaR grade.

Effect of eluents on antigen and antibody

Four potential eluents were assessed qualitatively and quantitatively for any

denaturing effects on human IgG antigen and sheep anti-human IgG antibody. The antigen (0.1 ml, 10.5 mg ml^{-1}) and antibody (0.1 ml, 24.7 mg ml^{-1}) were independently incubated at room temperature in 0.9 ml of the following eluents: ammonia solution (0.5 *M*, pH 11.5); glycine–HCl (0.2 *M*, pH 2.2); NaOH (2 m*M*, pH 11.5) and KSCN (3 *M*). Control antigen and antibody solutions consisted of 0.1 ml of each protein solution in 0.9 ml saline. The solutions were quantitated by the direct Mancini[18] and reverse Mancini techniques for the antigen and antibody respectively after incubation times 0.15 min, 30 min, 1 h, 4 h and 24 h.

Affinity constant determination

CNBr-activated Sepharose 4B–sheep anti-human IgG immunoadsorbent (9.87 mg anti-IgG per ml gel, binding capacity 0.6 mg IgG ml^{-1}) was prepared by the method of Kennedy and Barnes[2]. Equal portions (1 ml) were dispensed into 30-ml plastic universal containers containing 1.0 ml of each eluent: KSCN (3 *M*); NH_3 (0.5 *M*, pH 11.5) and glycine–HCl (0.2 *M*, pH 2.2). To each tube were added different known amounts of affinity purified human IgG. The containers were then capped and tumbled end over end at room temperature for 2 h. After centrifugation at 2000 *g*, duplicate samples of supernatants (4 μl) were pipetted into wells (3 mm diameter) in a Mancini gel plate (8 × 8 cm, containing 40 μl sheep anti-human IgG per 7 ml agar + 3% polyethylene glycol). Seward NIRDL serum standard was used for calibration. The plates were then left to develop in a moist atmosphere for 24 h. The plates were then washed for several hours in saline to remove soluble proteins, pressed-dried and stained with Coomassie Blue R250 and the precipitin ring diameters measured in two directions mutually at right angles. Quantitation was obtained from a standard curve produced from a plot of the square of the ring diameters of the standards against antigen concentration in mg ml^{-1}.

A Langmuir plot[12] of $1/r$ (r represents the molar ratio of antibody bound antigen molecules per total antibody immobilised) as ordinate against $1/c$ as abscissa (c represents the moles of free unbound antigen) yields a straight line which intercepts the ordinate at a value $1/n$ where n is the number of antibody binding sites (*i.e.*, the valency). The slope of the line is given by $1/Kn$ where K is the affinity constant. The experimental results are summarised in Table I and Fig. 3.

RESULTS AND DISCUSSION

Effect of eluents on antibody and antigen

The comparative analysis of four eluents revealed a noticeable decrease in the size of the direct Mancini precipitate rings after 24 h incubation with all four eluents (Fig. 1). There was, however, a more pronounced decrease in the precipitate ring size of the antigen compared with the antibody when both were incubated in glycine–HCl (0.2 *M*, pH 2.2). A plot of the percent decrease of precipitate ring diameter against incubation time revealed a 10% decrease in the antibody precipitate ring size after 24 h incubation in the glycine–HCl buffer (Fig. 2A). In contrast, there was a 56% decrease in the antigen precipitate ring size under the same conditions and the effect was a maximum after 1 h incubation. Sodium hydroxide (2 m*M*, pH 11.5) showed a small effect in reducing both antibody and antigen precipitate ring sizes (6% and 12%, respectively, after 24 h). With KSCN (3 *M*) there was a 20% decrease in antibody

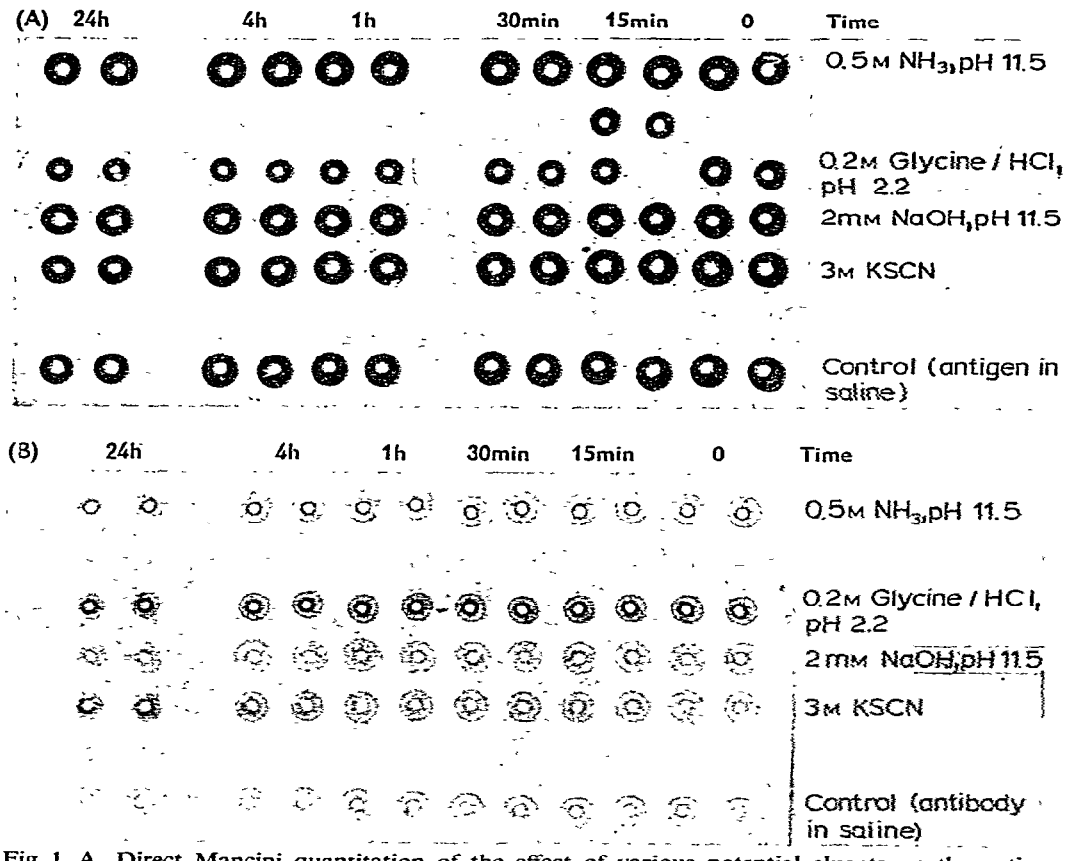

Fig. 1. A, Direct Mancini quantitation of the effect of various potential eluents on the antigen (human IgG). B, Reverse Mancini quantitation of the effect of various potential eluents on the antibody (sheep anti-human IgG).

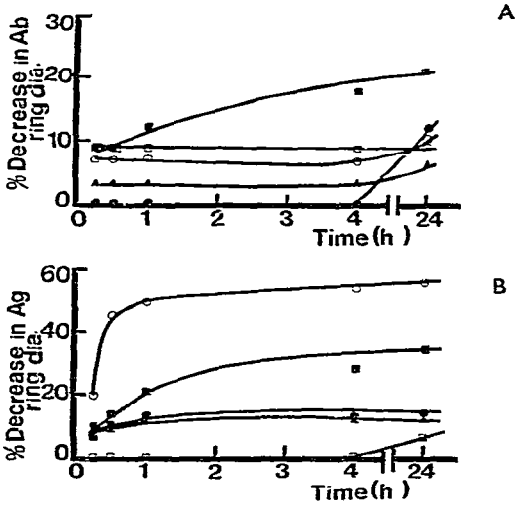

Fig. 2. Effect of four eluents on sheep anti-human IgG (A) and on human IgG (B). ▲, 2.0 m*M* NaOH, pH 11.5; □, 0.5 *M* NH_3, pH 11.5; ○, 0.2 *M* glycine–HCl; ■, 3 *M* KSCN; ●, control in saline.

precipitate ring diameter after 24 h compared with a 35% decrease in the antigen precipitate ring diameter over the same period. Ammonia solution (0.5 *M*, pH 11.5) had the least effect in reducing the antibody and antigen precipitate ring sizes after 24 h incubation (9% and 6%, respectively). However, there was no significant decrease in the antigen precipitate ring sizes after 4 h incubation (Fig. 2B).

In general, there was no appreciable difference between the 2 m*M* NaOH and the 0.5 *M* ammonia solutions in their effects on the antigen and antibody investigated. Of the four eluents compared, ammonia would be recommended by virtue of its apparent limited effect on the proteins after a 4 h contact time, its relative desorbing strength and ease of dialysis.

Determination of affinity constant

The desorbing power of eluents can be assessed from the affinity constant[23], *K*, derived from the slope of the straight line resulting from the Langmuir plots of the reciprocal of the ratio of the bound antigen to immobilised antibody, $1/r$, as ordinate against the reciprocal of the soluble antigen, $1/c$, as abscissa (Fig. 3).

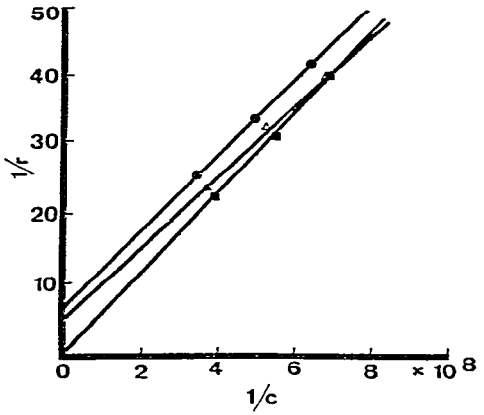

Fig. 3. Langmuir plot for the determination of affinity constants. ●, 3 *M* KSCN; △, 0.5 *M* NH_3; ■, 0.2 *M* glycine–HCl.

In this experiment the value of *n* was found to be 0.15 and 0.20 in KSCN (3 *M*) and NH_3 (0.5 *M*, pH 11.5) solutions, respectively. However, in glycine–HCl (0.2 *M*, pH 2.2) the value of *n* was 20 and the straight line appears to go through the origin. The number of binding sites for a pure, fully active, divalent antibody should theoretically be 2.0 (ref. 13). It is interesting to note that the experimentally determined value of 0.2 for the valence of the insolubilised antibody in ammonia solution compared best with the theoretical value. The most severe departure from the theoretical value was observed with the glycine–HCl system. Similarly, the experimentally derived affinity constant (Table I) was largest for the glycine system ($1.0 \cdot 10^{10}$) indicating that although it is a less powerful desorbing eluent than 3 *M* KSCN and 0.5 *M* ammonia solutions, the large value for *n* obtained with the glycine–HCl buffer would suggest that the antibody binding sites are more fully exposed and hence the avidity of the antibody could be potentiated[19]. Some evidence

TABLE I

SUMMARY OF QUANTITATIVE RESULTS FOR THE DETERMINATION OF AFFINITY CONSTANT OF THE REACTION OF IgG WITH ANTI-IgG–SEPHAROSE IMMUNOADSORBENT IN DIFFERENT ELUENTS

Eluent	*IgG added (nmoles)*	*IgG unbound, c (nmoles)*	*$10^{-8} \cdot 1/c$ ($mole^{-1}$)*	*IgG bound Ag (nmoles)*	*moles Ag/ moles Ab* coupled = r*	*1/r*	*K (M^{-1})*
3 *M* KSCN	5.3	2.90	3.4	2.40	0.039	25.6	
	3.8	1.98	5.0	1.82	0.029	34.5	
	3.0	1.56	6.4	1.44	0.023	43.5	$1.2 \cdot 10^8$
	1.5	1.13	8.9	0.37	0.006	166.7	
	0.4	nil					
0.5 *M* NH_3	5.3	2.70	3.7	2.60	0.042	23.8	
pH 11.5	3.8	1.90	5.3	1.90	0.030	33.3	
	3.0	1.44	6.9	1.56	0.025	40.0	$1.0 \cdot 10^8$
	1.5	1.13	8.9	0.37	0.006	166.7	
	0.4	nil					
Glycine–HCl	5.3	2.55	3.9	2.75	0.044	22.7	
(0.2 *M*, pH 2.2)	3.8	1.80	5.5	2.0	0.032	31.3	
	3.0	1.44	6.9	1.56	0.025	40.0	$1.0 \cdot 10^{10}$
	1.5	1.13	8.9	0.37	0.006	166.7	
	0.4	nil					

* Amount of anti-IgG coupled = 62.0 nmoles.

for such an hypothesis is observed in the relatively smaller decrease in precipitate ring diameter in the reverse Mancini system (Fig. 1A and B). Our affinity constant values are in good agreement with the values ($K = 3.3 \cdot 10^7$, $n = 0.35$ in phosphate buffer, 0.1 *M*, pH 8.0) reported by Eveleigh and Levy[20] for a human albumin–goat anti-(human albumin) system. The affinity constants determined (Table I) accord with the studies on the immunological integrity of human IgG and sheep antihuman IgG incubated in similar eluents over a period of 24 h (Figs. 1 and 2).

The significant reduction in the precipitate ring diameters, for the direct and reverse Mancini systems in the presence of glycine–HCl buffer (0.2 *M*, pH 2.2), corresponds with the affinity constant ($K = 1 \cdot 10^{10}$) determined in the same buffer system. This would suggest that although glycine–HCl should desorb less protein from the immunoadsorbent compared with the other eluents, its powerful denaturing effect could result in a reduction of the effective yield of eluted antigen. Although a high affinity constant theoretically reflects a strong antigen antibody avidity, the effect of a given eluent on the antigen and the antibody could be quite variable, and could therefore affect the free energy distribution of antibody receptors for the antigen ligands.

Levison *et al.*[21] demonstrated that the antibody binding site is relatively open and accessible to large antigens in the presence of chaotropic ions such as chloride, perchlorate and thiocyanate. This seems to be confirmed by the range of affinity constants determined. Although the thiocyanate ion is a more potent chaotrope than the chloride anion from their position in the lyotropic series of cations[22], the antibody binding sites appear to be more accessible in the presence of glycine–HCl.

The following more widely embracing conclusions which can be drawn from these results are of practical importance for the optimisation of immunoadsorption techniques.

(1) The adsorption efficiency is directly related to the antigen concentration; the higher the antigen concentration of the applied antigen the more efficiently it will be extracted. This is particularly significant in immunosubtractive applications where the removal of a particular protein is desired.

(2) Since the affinity constants are affected by strongly acidic and strongly alkaline eluents as well as by chaotropic solutions, the use of moderate affinity antibodies could afford a reduction in the eluent concentration and volume with possible increase in yields.

(3) A knowledge of affinity constant must be correlated with qualitative and quantitative assessments of eluents on antigens and antibodies.

Many fundamental properties such as specificity, reaction rate and affinity constant depend on the individual antibody antigen system explored. Nevertheless, the choice of support materials and the conditions of the experimental protocol are of cardinal importance for the successful operation of immunoadsorbent columns and these results present a simple but successful approach to the choice of eluents and hence the judicious optimisation of yield in immunoadsorption techniques.

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