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Review article

Kinetics of antigen-antibody reactions at solid-liquid interfaces

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The kinetics of antigen-antibody reactions is reviewed with special attention paid to the specific properties at solid-liquid interfaces. Theories of possible diffusion limitation in forward reaction rates are compared to experiments. It is found that the intrinsic forward reaction rate in the bimolecular antigen-antibody reaction is normally not limited by diffusion either in solution or at the solid-liquid interface. However, reactions at the solid-liquid interface can be diffusion limited due to depletion of reactants close to the surface. This effect depends on geometry, intrinsic reaction rate and surface concentration of receptor molecules. Normally cell surface reactions are not diffusion limited whereas reactions at artificial surfaces often are limited by diffusion. When not limited by diffusion it is also found that the intrinsic forward and reverse reaction rates are lower for surface reactions compared to reactions in solution. Antigen-antibody reactions at solid-liquid interfaces can often be considered as practically irreversible and limited by mass transport or steric interactions.

Key words: Antibody; Antigen; Kinetics; Solid phase; Immunoassay

Introduction

Many routine methods in immunology are based on immunochemical reactions carried out at a solid-liquid interface. This is mainly because solid-phase methods provide simple means of separating bound and free reactants. Solid-phase methods are also known to be sensitive and can be combined with different measuring techniques available for surface analysis. When interpreting the data obtained from assays some assumptions must be made about the kinetics of the antigen-antibody reaction at the solid-liquid interface. Specific knowledge about the kinetics of the antigen-antibody reaction at a solid-liquid interface is of vital importance when optimizing a solid-phase assay procedure or when chosing an appropriate mathematical expression for fitting a standard curve to empirical data. However, most experiments on the kinetics of antigen-antibody reactions have been carried out for reactions in solution or at the cell surface. In this review, the kinetics of antigen-antibody reactions is discussed with special attention to the specific properties at solid-liquid interfaces. Special interest is focussed on the possibility of finding theoretical models for the kinetics of different solid-phase methods like enzyme-linked immunosorbent assay (ELISA) or solid-phase radioimmunoassay (RIA).

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Kinetics of the antigen-antibody interaction

General considerations

The antigen-antibody interaction is known to be a very fast and specific bimolecular reaction (for general reviews see De Lisi, 1976; Berzofsky and Berkower, 1984). We can describe the antigen-antibody interaction as a bimolecular reaction between two reactants A and B according the formula

$$A+B \underset{k_{-0}}{\overset{k_0}{\rightleftharpoons}} \dots \underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} (A\cdot B)_i \dots \underset{k_{-n}}{\overset{k_n}{\rightleftharpoons}} AB \qquad (1)$$

Here k_i are the different forward reaction rates, k_i are the different reverse reaction rates and AB is the final complex formed after n different intermediates states $(A \cdot B)_i$. For multivalent reactants A or B the intermediate states $(A \cdot B)_i$ may represent states with different amounts of multiple binding.

Since we are dealing with very fast macromolecular reactions we must also consider possible diffusion control in the reaction. One way to do this is to identify the initial intermediate state $(A \cdot B)_0$ as a so called 'encounter complex' which must first be formed before the reaction, can continue. In this case the rate constant k_0 represents the diffusion rate constant with which the two reactants are appropriately positioned for reaction. It may involve both translational and rotational diffusion. Correspondingly the rate constant k_{-0} represents the reverse diffusion rate constant with which an encounter complex can dissociate.

In the above reaction scheme the different reaction rates are most probably very different in magnitude. Normally both the overall forward reaction rate and the overall reverse reaction rate are limited by one particular rate constant and the overall reaction scheme can be simplified to

$$A+B \underset{k_r}{\overset{k_f}{\rightleftharpoons}} AB \qquad (2)$$

where k_f is the effective forward reaction rate constant and k_r is the effective reverse reaction rate constant. By considering the intermediate states as quasi-stationary, simplified expressions for the overall rate constants can be obtained. This procedure of reducing a complicated reaction scheme has been used in many studies for example when analyzing bivalent binding (Perelson et al., 1980) and also when studying cell surface reactions (Dembo et al., 1979; DeLisi, 1981).

Forward reactions in solution

Let us examine under what circumstances the forward reaction rate k_f is limited by diffusion (for a review of diffusion-controlled macromolecular interactions see Berg and Von Hippel, 1985). For two uniformly reactive spheres A and B the bimolecular association constant is limited by a diffusion rate constant described by the Smoluchowski equation

$$k_0 = 4\pi(D_A + D_B)(r_A + r_B) = 4\pi DR \qquad (3)$$

where D_A and D_B are the diffusion constants, and r_A and r_B the radii of reactants A and B respectively. $D = D_A + D_B$ is the relative diffusion constant of the reactants and $R = r_A + r_B$ is the effective interaction radius. The different diffusion constants can be related to radii by the Stokes-Einstein relation

$$D_A = k_B T/6\pi\eta r'_A; \qquad D_B = k_B T/6\pi\eta r'_B \qquad (4)$$

where η is the fluid viscosity, k_B is the Boltzmann constant, T is absolute temperature and r'_A and r'_B are the hydrodynamic radii of reactants A and B respectively. For an order of magnitude analysis we can assume that the interaction radius is equal to the hydrodynamic radius and we get

$$k_0 = (2k_B T)(r_A + r_B)^2/3\eta r_A r_B = k_{0e} f_r \qquad (5)$$

where

$$k_{0e} = 8k_B T/3\eta \qquad (6)$$

and

$$f_r = (r_A + r_B)^2/4r_A r_B \qquad (7)$$

With this notation we have introduced a geometrical factor f_r and a fundamental rate constant k_{0e} which represents the diffusion rate constant for reactants of equal radius. For reactions in water at

room temperature we find that k_{0e} is approximately $7 \cdot 10^9$ $M^{-1}s^{-1}$. If the radii of the reactants differ, the diffusion association rate constant k_0 increases and for example with $r_A/r_B = 10$ we get $f_r \approx 3$ and $k_0 \approx 2 \cdot 10^{10}$ $M^{-1}s^{-1}$. These forward reaction rates are much higher than measured rates, even for the fastest hapten-antibody reaction in solution. From this type of approximate analysis it must be concluded that diffusion rates are not rate-limiting for antigen-antibody reactions in solution. Also steric factors and a possible activation barrier for reaction have to be considered (Bell and DeLisi, 1974).

One way to involve steric factors is to include rotational diffusion in the analysis (Schmitz and Schurr, 1972). For a reactive patch on a spherical molecule equation (3) has to be modified and we get (Berdnikov and Doktorov, 1982; Berg, 1985)

$$k_0 = 4\pi DR \sin(\theta_A/2) \tag{8}$$

where θ_A is the allowed reaction angle at molecule A. For small angles θ_A and letting the reactive site radius equal the smaller reactant radius r_B we get $\theta_A = r_B/r_A$ and

$$k_0 \approx 4\pi DRr_B/2r_A = k_{0e}\frac{1}{8}\left(\frac{r_B}{r_A}+1\right)^2 \tag{9}$$

For small ratios r_B/r_A the geometrical factor f_r is now smaller than one. With a reasonable relation between hapten and antibody radius of say $r_B/r_A = 0.2$ we get $f_r \approx 0.2$. Thus, when rotational diffusion is considered we can get diffusion rate constants of the order of $1 \cdot 10^9$ $M^{-1}s^{-1}$. This is quite close to the fastest measured hapten-antibody reactions in solution, around $6 \cdot 10^8$ $M^{-1}s^{-1}$. However, most hapten-antibody reactions are slower (Pecht and Lancet, 1976).

For macromolecular reactions where the reactive sites are small compared to the molecular dimensions the diffusion rates can be reduced further. Consider two macromolecules A and B, each with reactive areas described by the angles θ_A and θ_B. In this case the diffusion association rate constant is described by (Schurr, 1976):

$$k_0 = 4\pi DR\theta_A\theta_B(\theta_A + \theta_B)/8 \tag{10}$$

Letting r_s be the radius of the reactive site on each molecule and $r_B = r_A$ we get $\theta_A = \theta_B = r_S/r_A$ and also

$$k_0 = k_{0e}\frac{1}{4}\left(\frac{r_S}{r_A}\right)^3 \tag{11}$$

The geometrical factor is now further reduced so that for $r_S/r_A = 0.2$ the diffusion rate constant is of the order of $1 \cdot 10^7$ $M^{-1}s^{-1}$. From a collection of different forward reaction rates (Mason and Williams, 1986) it can be seen that antibody reactions with protein antigen approach this value in some cases but normally they are a few orders of magnitude lower.

To summarize this section we conclude that although antigen-antibody reactions are very fast, they are not limited by translational diffusion. However, when steric effects are considered in the form of rotational diffusion, measured forward reaction rates are sometimes of the same order of magnitude as predicted. One important aspect of diffusion macromolecular interactions is that it is the size of the reactive regions rather than the size of the reactants that determines the actual reaction rates (Doi, 1975; Berg and Von Hippel, 1985).

Flat surface

Let us focus our interest on surface reactions and continue the preceeding section by analysing the diffusion association reaction at a reactive site on a flat surface. The diffusion forward reaction rate to a reactive disc of radius r_S is (Hill, 1975)

$$k_0 = 4Dr_S \tag{12}$$

where r_S is the radius of the reactive site. If we also include the reduction in the effective capture radius by the constraint factor $\sin^2(\theta_A/2)$ due to a small reactive region on the associating molecule (Hill, 1975) we get

$$k_0 = 4Dr_S \sin^2(\theta_A/2) \tag{13}$$

Letting $\theta_A = r_S/r_A$ we get finally

$$k_0 = k_{0e}\frac{1}{16\pi}\left(\frac{r_S}{r_A}\right)^3 \tag{14}$$

From equations (9), (11) and (14) we can see what happens with the diffusion forward reaction rate

for a hapten-antibody reaction when in solution and when the hapten is immobilized on a protein carrier or surface. We can see that when the hapten is immobilized on a surface the diffusion forward reaction rate might well be reduced to $1 \cdot 10^6$ $M^{-1}s^{-1}$ (for $r_S/r_A = 0.2$).

For receptor sites immobilized on a surface the measured forward reaction rates can often be less than predicted by equation (14). If adjacent sites are closely packed their ability to bind reactants decreases the local concentration of reactants and the forward reaction rate is reduced. This effect becomes more pronounced as the surface concentration of receptors is increased. They are very important for artificial surfaces with high concentrations of immobilized receptors. With a high local concentration of receptors at a surface, the solution may well be depleted of reactants close that surface and reactants may have to diffuse over macroscopic distances before reaction. Apart from introducing new diffusion limitations the analysis must be carried out in a different way. The system may not be in a steady-state and before using the steady-state approximation we have to make a transient analysis.

Transient analysis of diffusive forward reaction rates at surfaces

So far we have seen that the intrinsic reaction between antigen and antibody is not limited by translational diffusion. Many collisions normally occur before the reactants are appropriately oriented or activated for reaction to take place. Let us now look at antigen-antibody reactions at surfaces where there may be high surface concentrations of reactive sites. We start by analysing the situation shown in Fig. 1. For the initial forward reaction rate we can write (Stenberg et al., 1986)

$$\frac{d\Gamma_A}{dt} = k_f c_s \Gamma_0 \qquad (15)$$

where Γ_A is the surface concentration of bound reactant, c_s is the concentration of reactants in solution close to the surface and Γ_0 is the surface concentration of reactive sites. In this relation we have used the intrinsic forward reaction rate k_f as a measure of maximum speed in the forward

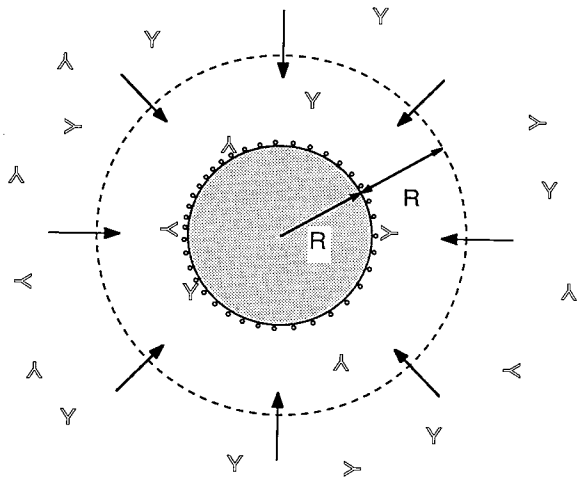

Fig. 1. Reaction between antibodies in solution with antigen immobilized on a sphere. The forward reaction is first limited by the surface reaction. After some time the solution is depleted of antibodies close to the surface and diffusion limitations can occur. A steady-state may develop where the antibodies diffuse across a diffusion barrier equal to the sphere radius.

reaction knowing that this rate may be limited by rotational diffusion or any rate limiting step in the association reaction (1) above. Since we are dealing with both volume and surface concentrations we must be aware of the different dimensions involved. For example, let Γ_A and Γ_0 be expressed in mol/cm^2, c_2 in mol/l (M) and k_f in l/mol-s ($M^{-1}s^{-1}$).

We study the case where the reactive sites are immobilized at the surface of a sphere of radius R and where the sphere is introduced into an unstirred solution at time zero. In solution the transport of reactants follow the diffusion equation and at the surface the antigen-antibody reaction follows equation (15). By combining the diffusion equation in solution with appropriate boundary and initial conditions at the surface an expression for the amount bound can be found (see appendix). From this model calculation some conclusions may be drawn. The amount bound is proportional to the initial concentration of reactants, c_0, and also depends on both geometry and time. A useful parameter describing the degree of diffusion control in the reaction is the so called Damkoehler number, D_a, defined by

$$D_a = \frac{Rk_f\Gamma_0}{D} \qquad (16)$$

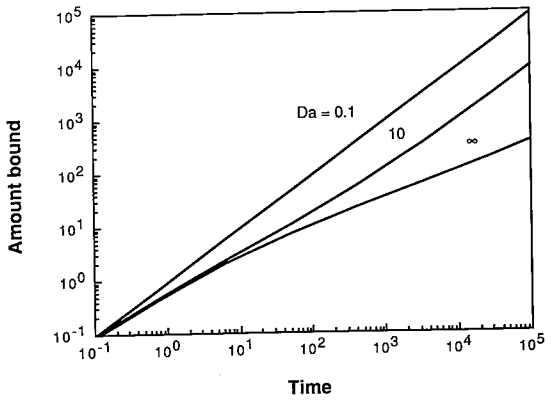

Fig. 2. Theoretically calculated amount bound in the forward reaction as described in Fig. 1. The amount bound and the time are normalized (see text) and the parameter is the Damkoehler number D_a which is a measure of diffusion limitation in the forward reaction. The limit $D_a \rightarrow \infty$ corresponds to a plane surface.

where D is the diffusion constant of the diffusing reactant and R is the radius of the sphere with immobilized reaction sites. In this relation there is no bulk concentration so we have to be careful about the dimensions. If Γ_0 is expressed in moles per cm^2, R in cm and D in cm^2/s we must have k_f expressed in ml/mol-s instead of 1/mol-s.

Some binding curves from the theoretical model are shown in Fig. 2. The amount bound is normalized and given as $(\Gamma_A k_f \Gamma_0)/(c_0 D)$ where c_0 is the initial concentration of antibodies in solution. Time is also normalized and given as t/τ where τ is a time constant defined below. The Damkoehler number D_a can be interpreted as the ratio between the maximum reaction rate at the surface and the maximum rate of diffusional mass transfer across a water film of thickness R. A small Damkoehler number means that the surface reaction is slow compared to the diffusional mass transfer. A high Damkoehler number means that the surface reaction is fast compared to the diffusional mass transfer and that diffusion is rate limiting. It is important to note that apart from the diffusion constant, both the geometry (R) and the surface concentration of reactive sites (Γ_0) are involved in this parameter. From Fig. 2 we can see that it is only for D_a values greater than 1 that the binding is reduced compared to the intrinsic reaction.

To describe the time dependence it was found that a useful parameter was the following time constant,

$$\tau = D/(k_f \Gamma_0)^2 \tag{17}$$

It describes the time at which the reaction changes from an initial reaction control to a possible diffusion control. From Fig. 2 we can see that it is only for times longer than this time constant that diffusion limitations occur. Then the solution close to the surface is depleted from reactants and the reactants have to diffuse over longer distances before reaction. The particle radius is not involved in this time constant. However, the particle radius is involved in a second time constant

$$\tau_s = \frac{R^2 D_a^2}{D(1+D_a)^2}\frac{4}{\pi} \tag{18}$$

describing the time after which the binding is characterized by a steady-state situation. After a time characterized by this time constant the amount bound is proportional to time regardless of reaction or diffusion control in the reaction. From Fig. 2 we can see that for a D_a value of 10 the reaction is in a steady state after a normalized time of about 100. Even for high Damkoehler numbers and possible diffusion control in the reaction this time constant is quite short for cell surface reactions due to the small cell radius. For macromolecules reacting on cell surfaces this time constant is of the order of 0.2 s (Stenberg et al., 1986). However, for reactions on macroscopic particles used for immunoassays the time constant τ_s may well be of the order of minutes indicating that a transient analysis is appropriate.

After steady-state has developed the reaction rate on a sphere may be reduced by diffusion and equation (15) modified to (Stenberg et al., 1986)

$$\frac{d\Gamma_A}{dt} = \frac{k_f c_0 \Gamma_0}{1+D_a} \tag{19}$$

For high values of the Damkoehler number equation (19) is simplified to

$$\frac{d\Gamma_A}{dt} = \frac{D c_0}{R} \tag{20}$$

showing that in the diffusion limited region the rate is inversely proportional to the sphere radius.

Forward reaction at the cell surface

The forward diffusion reaction rate constant to reactive sites distributed over a spherical cell can be described by (Berg and Purcell, 1977; DeLisi, 1981)

$$k_0 = 4\pi DR\frac{Nr_S}{\pi a + Nr_S} \tag{21}$$

where a is the cell radius, and N is the number of reactive sites of radius r_s per cell. As before (equation 3) D and R describe the relative diffusion constant and capture radius. However, it is important to note that this rate constant relates to the whole cell and not to the individual sites. For a small ligand binding to a cell the relative diffusion constant D is dominated by the diffusion constant of the ligand and the capture radius R is dominated by the cell radius. Dividing equation (21) by N we get the rate per immobilized reaction site and we can compare equations (19) and (21). The effective forward reaction rate in equation (19) is $k_f/(1 + D_a)$ and after identification with equation (21) letting $R = a$ we get $k_f = 4Dr_S$ and $D_a = Nr_S/\pi a$. At first glance equation (21) gives the impression that the rate is constant or increases with cell radius. However, at constant surface concentration of reactive sites the diffusion rates decrease with increasing radius (equation 20).

In an experimental situation the time dependence is the same for diffusion or reaction control in the reaction after steady-state has developed. One way to determine whether there is reaction or diffusion control in an actual experiment is to calculate the so called observable Damkoehler number ηD_a defined by

$$\eta D_a = \frac{\Gamma_A R}{Dc_0 t} \tag{22}$$

which is the ratio between measured rate and maximum diffusion limited rate according equation (20). In the steady-state, values of ηD_a less than one indicate reaction control of the reaction. For cell surface reactions this type of analysis normally indicates reaction control of the reaction (Jerne et al., 1974; Wank et al., 1983). Thus, we can compare our predicted values of the diffusive association constant to individual sites (equations 12, 13 and 14) with measured forward reaction rates. These are normally in the range 10^5–10^6 $M^{-1}s^{-1}$ for reactions between antibody and cell surface receptors (Davie and Paul, 1972; Mason and Williams, 1980; Wank et al., 1983; Roe et al., 1985). Only after considering rotational diffusion (equation 12) the associative rates are of this order of magnitude for surface immobilized receptors. We can conclude that also for cell surface reactions the intrinsic forward reaction is not limited by translational diffusion. From equation (16) we can calculate what receptor density is needed to get a Damkoehler number of the order of one which is the limit where translational diffusion to the cell surface will start to influence the overall forward reaction. With an intrinsic forward reaction rate k_f of the order of 10^6 $M^{-1}s^{-1}$ it is only for very high receptor numbers of the order of 10^6 per cell that the reaction becomes diffusion controlled.

Forward reaction at artificial surfaces

Let us now examine the forward reaction when receptors are immobilized at macroscopic spheres or flat surfaces. For a given intrinsic forward reaction rate k_f, diffusion constant D and surface concentration Γ_0 both the Damkoehler number D_a and the time constant τ_s increase with the radius of the immobilizing particle. To get an order of magnitude estimate of the different parameters we continue the analysis by using equations (16), (17) and (18). After adsorption of a protein layer or covalent bonding of receptor molecules there will most probably be a receptor site concentration in the range 10^{-11}–10^{-12} mol/cm^2. With a forward reaction rate k_f of at least 10^5 $M^{-1}s^{-1}$ and a diffusion constant D of $4 \cdot 10^{-7}$ cm^2/s (a representative value for an IgG antibody or protein antigen) we find that the Damkoehler number is greater than one already for spheres more than 40 μm in diameter. For particles with a diameter of 1 mm the time constants τ_s is of the order of 1 h. We also find that the time constant τ is easily less than 1 min. All these figures show that for artificial receptor-coated surfaces there will most prob-

ably be diffusion limitation in the forward reaction.

At a plane surface the binding reaction is eventually limited by diffusion and there will be no steady-state in an unstirred solution. This happens for times longer than the time constant τ above. Then the binding can be approximated by a simplified expression describing diffusion limited association at a plane surface:

$$\Gamma_A = \frac{2}{\sqrt{\pi}} c_0 \sqrt{Dt} \qquad (23)$$

One important difference between equation (23) and equation (15) is that the binding is no longer proportional to time but rather to the square-root of time. This effect is also shown in Fig. 2, where it can be seen that the binding is proportional to the square-root of time for times greater than τ and for $D_a \rightarrow \infty$. Equation (23) has long been used in studies on surface adsorption of proteins and enzymes for example (Trurnit, 1954). It has also been found to be adequately accurate in many experimental studies on antigen-antibody reactions at plane surfaces (Nygren and Stenberg, 1985a, b; Nygren et al., 1985, 1987). If this relation is valid there is well defined relation between amount the bound and the initial concentration of reactants. In some cases the binding was found to follow equation (23) in the initial binding up to surface concentrations of around 1 pmol/cm^2 (Nygren et al., 1987). After checking that the relation is valid it can also be used as a quantitative tool to calibrate the ELISA (Nygren and Stenberg, 1985b). An example is shown in Fig. 3 where equation (23) has been used to calculate the surface concentration of antibodies for different incubation times and initial concentrations of antibodies in solution.

Equation (23) can be derived by solving the diffusion equation assuming that all reactants reaching the surface are bound. Mathematically this is accomplished by introducing the boundary condition of zero concentration close to the surface. The technique has been found to be very useful in many experimental systems such as radial diffusion of antibodies over an antigen-coated surface (Stenberg et al., 1982), diffusion of cholera-toxin over a receptor-coated surface (Stenberg and Nygren, 1982) and binding of antibodies at a receptor-coated surface after diffusion through a thin gel layer (Stenberg and Nygren, 1985). In these cases different solutions to the diffusion equation have been used depending on the different geometries involved in the experimental systems. The diffusion and thus the binding equation is geometry dependent which often results in quite complicated mathematical expressions. Simplified expressions can often be derived as illustrated by the finding that for the specific geometry of a microtiter plate, equation (23) is adequately accurate in some early time domains (Stenberg et al., 1988). However, one prerequisite for using this mathematical technique is that the reaction not be limited for other reasons such as lack of receptor sites. Neither can the binding be influenced by the reverse reaction in the binding reaction. Thus the technique is valid only for practically irreversible reactions at the surface. Our early studies showed that the antibody binding to surface immobilized antigen could be regarded as practically irreversible (Stenberg et al., 1982). This leads to a detailed investigation of the reverse reaction in the antigen-antibody interaction.

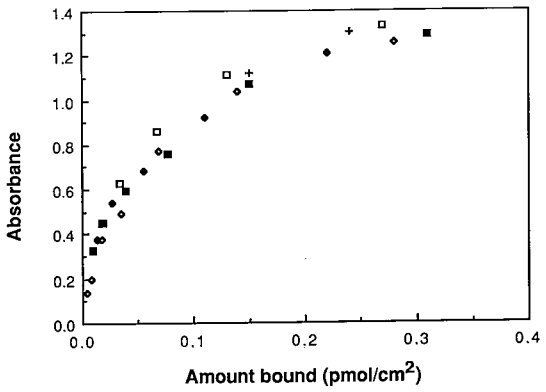

Fig. 3. Calibration curve for a microtiter plate ELISA. Absorbance values are plotted versus surface concentration of monoclonal antibody. (+) Surface concentration experimentally determined by ellipsometry. Surface concentration from theoretical model and incubation time: (□) 15 min, (◆) 10 min, (■) 5 min, (◇) 1 min. From Stenberg et al. (1988).

Reverse reaction rate

For a hapten-antibody interaction in solution the reverse reaction rate is normally considered to determine the strength of the binding. The dissoci-

ation rate constant may vary considerably between different hapten and antibody combinations. However, it has been found that haptens can be divided into two groups, rapidly and slowly dissociating haptens. The reverse rate constant for rapidly dissociating haptens is normally of the order of one/s or larger whereas the reverse rate constant for slowly dissociating haptens is less than 10^{-3} s^{-1} (Pecht and Lancet, 1976). For cell surface reactions with protein antigens or antibodies the reverse rate constant is normally less than 10^{-3} s^{-1} and not seldom of the order of 10^{-5} s^{-1} (Mason and Williams, 1986). The dissociation of antibodies from receptors immobilized at artificial surfaces also has rate constants in the range 10^{-4}–10^{-5} s^{-1} (Li, 1985; Nygren et al., 1985). With dissociation rate constants of this order of magnitude there is no problem in carrying out experiments during a time scale where the binding can be regarded as irreversible.

Experimental studies show that the reverse reaction rate is considerably lower for surface reactions than for fast hapten dissociation in solution. This effect has often been explained by the kinetic effects of polyvalence in the antigen-antibody reaction (Crothers and Metzger, 1972). Theoretically, the reverse rate could be decreased by several orders of magnitude due to multiple binding. However, many experimental studies on Fab fragments as well as bivalent IgG antibodies show that the dissociation rate is very low even for monovalent antibodies (Mason and Williams, 1980; Nygren et al., 1985, 1987). The mechanism behind this slow dissociation has been suggested to be mass transport limitation of the reverse reaction (Berzofsky and Berkower, 1984). With a high surface concentration of receptors there is a high probability of reassociation instead of diffusion transport away from the surface after a dissociation. Similar effects have been found for ligands diffusing away from a dialysis bag with ligand binding protein present in the bag (Silhavy et al., 1975).

For a surface reaction it can be shown that it is the magnitude of the Damkoehler number, D_a, that governs the relative importance of reassociation. With a high D_a value there is a high probability of reassociation before escape from the surface. Previously we have seen that normally the D_a values are less than one for cell surface reactions but can be greater than one for reactions carried out on macroscopic spheres.

Controlled experiments on diffusional mass transfer should be carried out in a well defined hydrodynamical situation for example by using a rotating disc commonly used in electrochemistry (Opekar and Beran, 1976). Well defined boundary diffusion layers can be obtained using this technique. A rotation speed of 1500 rev/min should give a boundary thickness for macromolecules of the order of 10 μm. Experiments on dissociation of monoclonal antibodies from surface immobilized antigen failed to show any significant increase in dissociation under these conditions in PBS buffer (Nygren et al., 1987). Only with antigen present in excess in solution was there any significant dissociation of monoclonal antibody. It was found that the hapten itself was more effective in promoting dissociation than hapten immobilized on a carrier protein. However, the initial dissociation of Fab fragments was shown to depend on stirring and also was the dissociation found to be more rapid than the dissociation of the corresponding divalent monoclonal. Thus, both multivalence and mass transfer from the surface can influence the dissociation as predicted.

Affinity and equilibrium analysis

In many situations there is an interest in using the ultimate binding strength of the immobilized receptor molecules to get sensitive assays. Sensitivity can be improved by increasing the incubation time until the binding is limited by the onset of the reverse reaction. Eventually there is equilibrium between the forward reaction and the reverse reaction. Then the binding is limited by the binding strength or affinity normally defined by the equilibrium constant

$$K_a = \frac{k_f}{k_r} \tag{24}$$

For an equilibrium analysis we must also consider the limited number of receptor sites at the surface. With this in mind we can formulate the rate of the antigen-antibody reaction at a solid-liquid inter-

face (Nygren et al., 1986)

$$\frac{d\Gamma_A}{dt} = k_f c_s (q\Gamma_{Ag} - p\Gamma_A) - k_r \Gamma_A \quad (25)$$

where Γ_{Ag} is the surface concentration of antigen at the surface, q is the number of epitopes per antigen molecule and p is the number of blocked epitopes per bound antibody molecule. The parameter p can be compared to the obstruction factor introduced in the analysis of cell receptor kinetics (Mason and Williams, 1980; Roe et al., 1985). With our notation we find that the maximum amount bound is $(q/p)\Gamma_{Ag}$. At equilibrium $(d\Gamma_A/dt = 0)$ equation (25) can be reformulated to obtain the so called Scatchard equation

$$\frac{\Gamma_A}{c_s \Gamma_{Ag}} = K_a' \left(\frac{q}{p} - \frac{\Gamma_A}{\Gamma_{Ag}} \right) \quad (26)$$

where we have introduced an effective association constant

$$K_a' = p \frac{k_f}{k_r} \quad (27)$$

With this definition $1/K_a'$ is the antibody concentration at which the binding Γ_A has reached half its maximum binding. If experiments follow equation (25) a plot of $(\Gamma_A/c_s\Gamma_{Ag})$ versus (Γ_A/Γ_{Ag}) should give a straight line and the possibility of evaluating both K_a' and q/p.

A set of different monoclonal antibodies was examined by ellipsometry and the data was analyzed by this technique (Nygren et al., 1986). It was shown that for monoclonal antibodies with homogeneous affinity constants in the range 10^5–$10^7\ M^{-1}$ affinity increased when haptens were immobilized at a surface by a carrier protein. However, for the surface reactions the different clones differ not only in the reverse rate constant but also in the forward rate constant and in the above parameter q/p. These findings indicate that for close packed antigen layers at artificial surfaces the manner in which the epitopes are presented may be important. This leads us to investigate structural aspects of the interaction layer.

Structural aspects of antigen-antibody binding

X-ray crystallographic studies of ligand-antibody complexes can give detailed information about the molecular basis for antigen-antibody interaction. In some cases the active site has been described in detail both regarding polypeptide sequence and conformation (Mariuzza et al., 1987). From these studies we note that the size of the active site of a Fab-lysozyme complex is approximately 2×3 nm, giving support for our estimate of r_S/r_A above. Structural aspects of antigen-antibody interaction have also been studied on two-dimensional crystals by electron microscopy (Reidler et al., 1986). These investigations have shown that already at surface concentrations of 0.8 pmol/cm^2 the antibodies can interact in a well defined hexagonal pattern (Uzgiris and Kornberg, 1983). Structural aspects in the layer can often impose restrictions on the forward binding kinetics as well as steric limitations. Much knowledge in this area has developed from X-ray crystallographic studies and so far little has been done to incorporate this knowledge into kinetic studies. Experimental studies on the binding of a second layer to a first layer of adsorbed organic molecules have shown that the binding capacity goes through a maximum with increased surface concentration in the first layer (Elwing et al., 1984). Simple geometrical arguments may lead to mathematical models which can qualitatively account for these effects at high surface concentrations in the second layer (Lundström and Elwing, 1984). Kinetic studies in connection with electron microscopic investigations have shown that steric interactions at surface concentrations around 1 pmol/cm^2 can influence the binding kinetics (Nygren et al., 1987). All these findings show that steric limitations and lack of available reactive sites may well limit the binding of a biospecific ligand to a surface immobilized receptor, and not the onset of a reverse reaction. With this in mind we now analyse some of the results on solid phase immunoassays.

Kinetics of solid-phase immunoassays

Most methods used to study the kinetics of antigen-antibody reactions have so far been appli-

cable to small antigens such as hormones or peptides (as reviewed by Steward, 1986). For immunoassays where the analyte is a small hapten, equilibrium analysis is appropriate and the kinetics follow the law of mass action. Radioimmunoassay (RIA) was originally developed for this type of analysis (Berson and Yalow, 1959; Rodbard et al., 1971) and today most RIA data are interpreted the same way (Bolton and Hunter, 1986). Also for enzyme immunoassays (EIA) the kinetics have so far been considered to follow the law of mass action (Nakamura et al., 1986) as well as the sensitivity of solid-phase ELISA (Griswold, 1987). However, as we have seen above, antigen-antibody reactions at solid surfaces dissociate only slowly. In consequence most immunoassays performed with incubation times of a few hours are not in equilibrium. When plotted in a Scatchard plot also irreversible reactions give practically straight lines and apparent association constants (Mason and Williams, 1986). Before interpreting the data as equilibrium data it must always be checked whether the binding is independent of time and also whether the association constant is consistent with the association constant calculated from the forward and reverse reaction rates. For reactions at the solid-liquid interface it is normally hard to obtain this correlation (Nygren et al., 1986).

In the kinetic analysis there is often a discussion about the importance of affinity in the assay performance (reviewed by Steward and Lew, 1985). It has often been argued that the affinity parameter reflects behaviour for high OD values in the absorbance reading whereas the end point titre reflects analyte concentration (Peterfy et al., 1983). It has also been demonstrated that these effects are strongly affected by the epitope density of the immobilized receptors (Lew, 1984). Endpoint titres may be influenced by antibody affinity especially with antigens of low epitope density (Nimmo et al., 1984).

Measurements of radioactively labelled antibody in microtiter plates have shown that the binding of primary antibody is linear over at least three orders of magnitude (Koertge and Butler, 1985). The data in the upper part of the sigmoidal ELISA titration plot was explained as the result of steric hindrance in the detection system. The enzyme kinetics was shown to vary depending on the conjugate used. It is interesting to note how high the binding capacity can be for polyclonal antibodies binding to a first layer of antibodies at the surface. From ELISA calibration data (Nygren and Stenberg, 1985b; Stenberg et al., 1988) we can analyse the initial slope of absorbance versus surface concentration of the first antibody layer. The absorbance was shown to reach a plateau for increased surface concentration in the first layer as shown in Fig. 3. If we assume that this plateau in absorbance is related to steric hindrance at about 1 pmol/cm^2 we find that initially there are at least 20 conjugate molecules on each antibody. Other experimental findings support this high stoichiometric relation between second and first layer. For a diffusion-in-gel thin layer immunoassay (DIG-TIA) carried out on an antigen-coated surface the reaction zone sizes were found to increase after incubation with a second antibody (Elwing and Nilsson, 1980). Two concentrations of second antibody were tested and for the high concentration the increase in reaction zone size was found to correspond to a factor of 16 in initial concentration. Since the surface concentration at the detection level is proportional to initial concentration (Stenberg et al., 1982) we conclude that the second antibody layer binds with at least 16 molecules on each first layer antibody. From ellipsometric experiments on binding capacity we can also see that for polyclonal antibody binding to a first layer of antibody with varying surface concentration the initial stoichiomentric relation is of the order of 20 (Lundström and Elwing, 1984).

Experimental studies on binding characteristics of immunoglobulins to monoclonal and polyclonal capture antibodies adsorbed on plastic microtiter plates have shown that for long incubation times the amount bound could sometimes extend to up to 80% of the added amount (Butler et al., 1986). When the depletion of reactants from the well is of this order of magnitude a mass transfer analysis is appropriate before applying the law of mass action (Stenberg et al., 1988). A quantitative measure of the analyte concentration can also be achieved in a dynamic situation where the amount bound is time and concentration dependent. We have seen that surprisingly often the results obtained by ELISA could be explained by an analy-

sis of the mass transport limitation (Nygren and Stenberg, 1985a, b).

Diffusional limitations in ELISA performance have been considered for hemispherical antigen-coated wells (Li, 1985). After diffusion was considered the rate constants in the bimolecular surface reaction could be evaluated as well as the affinity constant. It was found that both the forward rate and the reverse rate are very low. The forward rate was found to be $8.8 \cdot 10^3$ M^{-1} s^{-1} and the reverse rate was found to be $2.5 \cdot 10^{-4}$ s^{-1}. These rates are comparable to what was found in ellipsometric studies on monoclonal antibodies binding to surface immobilized antigen (Nygren et al., 1986). Going to a surface immobilized system seems to induce both a decreased association rate as well as a decreased dissociation rate and normally there will be a net increase in overall affinity constant. The increased sensitivity of solid-phase ELISA permits the detection of very small concentrations and it has actually been used for indirect measurements of homogeneous association constants has high as 10^9 M^{-1} (Friguet et al., 1985).

Conclusion

When trying to exploit the inherent sensitivity and specificity of antigen-antibody reactions at artificial surfaces we must be able to describe the kinetics of antigen-antibody reactions at the solid-liquid interface. As we have seen there are some differences to be expected when going from homogeneous reactions to heterogeneous reactions at artificial surfaces. Both geometry and organization of receptor molecules at the surface induce new restrictions on the surface reaction. For macromolecules reacting at the solid-liquid interface there will be new diffusion restrictions due to cooperativity among the immobilized receptors and macroscopic distances to overcome before reaction. Mass transport aspects are important when optimizing macromolecular solid-phase assay kinetics. In this field there is a lot of knowledge to be gained from chemical engineering, especially concerning hydrodynamic properties and ways to improve mass transport to the active sites at the surface.

So far, most data on antigen-antibody interactions has been collected from homogeneous reactions and cell surface reactions. As we have seen this data is not always directly applicable to measurements at the solid-liquid interface. A further understanding of the explicit differences should provide better means of predicting optimal conditions for immunoassays.

Appendix

Using quantities defined in the text, the analytical expression for the amount bound in Fig. 1 is (Stenberg et al., 1986)

$$\Gamma_A = \frac{c_0 D}{k_f \Gamma_0} \frac{D_a^3}{(1+D_a)^3} \left[\frac{t^*}{D_a} + 2\sqrt{\frac{t^*}{\pi}} + \exp(t^*)\,\mathrm{erf}(\sqrt{t^*}) - 1 \right]$$

where the normalized times t^* and $\bar{t}$ are related to incubation time t by

$$t^* = \left(\frac{1+D_a}{D_a}\right)^2 \bar{t} \quad \text{and} \quad \bar{t} = \frac{t}{\tau}$$

References

Bell, G.I. and DeLisi, C. (1974) Antigen binding to receptors on immunocompetent cells. I. Simple models and interpretation of experiments. Cell. Immunol. 10, 415.

Berdnikov, V.M. and Doktorov, A.B. (1982) Steric factor in diffusion-controlled chemical reactions. Chem. Phys. 69, 205.

Berg, H.C. and Purcell, E.M. (1977) Physics of chemoreception. Biophys. J. 20, 193.

Berg, O.G. (1985) Orientation constraints in diffusion-limited macromolecular association. Biophys. J. 47, 1.

Berg, O.G. and Von Hippel, P.H. (1985) Diffusion-controlled macromolecular interactions. Ann. Rev. Biophys. Chem. 14, 131.

Berson, S.A. and Yalow, R.S. (1959) Quantitative aspects of the reaction between insulin and insulin-binding antibody. J. Clin. Invest. 38, 1996.

Berzofsky, I.A. and Berkower, I.J. (1984) Antigen-antibody interaction. In: W.E. Paul (Ed.), Fundamental Immunology. Raven Press, New York, p. 595.

Bolton, A.E. and Hunter, W.M. (1986) Radioimmunoassay and related methods. In: D.M. Weir (Ed.), Handbook of Experimental Immunology, Vol. 1. Blackwell, Oxford, pp. 26.1–26.56.

Butler, J.E., Spradling, J.E., Suter, M., Dierks, S.E., Heyermann, H. and Peterman, J.H. (1986) The immunochemistry

of sandwich ELISAs I. The binding characteristics of immunoglobulins to monoclonal and polyclonal capture antibodies adsorbed on plastic and their detection by symmetrical and asymmetrical antibody-enzyme conjugates. Mol. Immunol. 23, 971.

Crothers, D.M. and Metzger, H. (1972) The influence of polyvalence on the binding properties of antibodies. Immunochemistry 9, 341.

Davie, J.M. and Paul, W.E. (1972) Receptors on immunocompetent cells. IV. Direct measurement of avidity of cell receptors and cooperative binding of multivalent ligands. J. Exp. Med. 135, 643.

DeLisi, C. (1976) Antigen-antibody interactions. In: Lecture Notes in Biomathematics, Vol. 8. Springer-Verlag, Berlin, pp. 1–142.

DeLisi, C. (1981) The effect of cell size and receptor density on ligand-receptor reaction rate constants. Mol. Immunol. 18, 507.

Dembo, M., Goldstein, B., Sobotka, A.K. and Lichtenstein, L.M. (1979) Histamine release due to bivalent penicilloyl haptens: The relation of activation and desensitization of basophils to dynamic aspects of ligand binding to cell surface antibody. J. Immunol. 122, 518.

Doi, M. (1975) Theory of diffusion-controlled reaction between non-simple molecules. II. Chem. Phys. 11, 115.

Elwing, H. and Nilsson, L.-Å. (1980) Diffusion-in-gel thin layer immunoassay (DIG-TIA). Optimal conditions for quantitation of antibodies. J. Immunol. Methods 38, 257.

Elwing, H., Dahlgren, C., Harrison, R. and Lundström, I. (1984) Complement factor adsorption on solid surfaces- an ellipsometric method for investigation of quantitative aspects. J. Immunol. Methods. 71, 185.

Friguet, B., Chaffotte, A.F., Djavadi-Ohaniance, L. and Goldberg, M.E. (1985) Measurements of the true affinity constant in solution of antigen-antibody complexes by enzyme-linked immunosorbent assay. J. Immunol. Methods 77, 305.

Griswold, W.R. (1987) Theoretical analysis of the sensitivity of the solid phase antibody assay (ELISA). Mol. Immunol. 24, 1291.

Hill, T.L. (1975) Effect of rotation on the diffusion-controlled rate of ligand-protein association. Proc. Natl. Acad. Sci. U.S.A. 72, 4918.

Jerne, N.K., Henry, C., Nordin, A.A., Fuji, H., Koros, A.M.C. and Lefkovits, I. (1974) Plaque forming cells: Methodology and theory. Transplant. Rev. 18, 130.

Koertge, T.E. and Butler, J.E. (1985) The relationship between the binding of primary antibody to solid-phase antigen in microtiter plates and its detection by the ELISA. J. Immunol. Methods 83, 283.

Lew, A.M. (1984) The effect of epitope density and antibody affinity on the ELISA as analysed by monoclonal antibodies. J. Immunol. Methods 72, 171.

Li, C.K.N. (1985) ELISA-based determination of immunological binding constants. Mol. Immunol. 22, 321.

Lundström, I. and Elwing, H. (1984) Stereospecific binding capacity of proteins on surfaces. Simple mathematical models. J. Theor. Biol. 110, 195.

Mariuzza, R.A., Phillips, S.E.V. and Poljak, R.J. (1987) The structural basis of antigen-antibody recognition. Ann. Rev. Biophys. Biophys. Chem. 16, 139.

Mason, D.W. and Williams, A.F. (1980) The kinetics of antibody binding to membrane antigens in solution and at the cell surface. Biochem. J. 187, 1.

Mason, D.W. and Williams, A.F. (1986) Kinetics of antibody reactions and the analysis of cell surface antigens. In: D.M. Weir (Ed.,), Handbook of Immunology, Vol. 1. Blackwell, Oxford, pp. 38.1–38.17.

Nakamura, R.M., Voller, A. and Bidwell, D.E. (1986) Enzyme immunoassays: heterogeneous and homogeneous systems. In: D.M. Weir (Ed.), Handbook of Experimental Immunology, Vol. 1. Blackwell, Oxford, pp. 27.1–27.20.

Nimmo, G.R., Lew, A.M., Stanley, C.M. and Steward, M.W. (1984) Influence of antibody affinity on the performance of different antibody assays. J. Immunol. Methods 72, 177.

Nygren, H. and Stenberg, M. (1985a) Kinetics of antibody-binding to surface-immobilized antigen: influence of mass transport on the enzyme-linked immunosorbent assay (ELISA). J. Colloid Interface Sci. 107, 560.

Nygren, H. and Stenberg, M. (1985b) Calibration by ellipsometry of the enzyme-linked immunosorbent assay. J. Immunol. Methods 80, 15.

Nygren, H., Czerkinsky, C. and Stenberg, M. (1985) Dissociation of antibodies bound to surface-immobilized antigen. J. Immunol. Methods 85, 87.

Nygren, H., Kaartinen, M. and Stenberg, M. (1986) Determination by ellipsometry of the affinity of monoclonal antibodies. J. Immunol. Methods 92, 219.

Nygren, H., Werthén, M. and Stenberg, M. (1987) Kinetics of antibody binding to solid-phase-immobilized antigen. Effect of diffusion rate limitation and steric interaction. J. Immunol. Methods 101, 63.

Opekar, F. and Beran, P. (1976) Rotating disc electrodes. J. Electroanal. Chem. 69, 1.

Pecht, I. and Lancet, D. (1976) Kinetics of antibody-hapten interactions. In: I. Pecht and R. Rigler (Eds.), Chemical Relaxation in Molecular Biology. Springer-Verlag, Heidelberg, pp. 306–338.

Perelson, A.S., Goldstein, B. and Rocklin, S. (1980) Optimal strategies in immunology. III. The IgM-IgG switch. J. Math. Biol. 10, 209.

Peterfy, F., Kussela, P. and Mäkelä, O. (1983) Affinity requirements for antibody assays mapped by monoclonal antibodies. J. Immunol. 130, 1809.

Reidler, J., Uzgiris, E.E. and Kornberg, R.D. (1986) Two-dimensional crystals of immunoglobulins. In: D.M. Weir (Ed.), Handbook of Experimental Immunology, Vol. 1. Blackwell, Oxford, pp. 17.1–17.5.

Rodbard, D., Ruder, H.J., Vaitukaitis, J. and Jacobs, H.S. (1971) Mathematical analysis of kinetics of radioligand assays: improved sensitivity by delayed addition of labeled ligand. J. Clin. Endocrinol. 33, 343.

Roe, R., Robins, R.A., Laxton, R.R. and Baldwin, R.W. (1985) Kinetics of divalent monoclonal antibody binding to tumour cell surface antigens using flow cytometry: standardization and mathematical analysis. Mol. Immunol. 22, 11.

Schmitz, K.S. and Schurr, J.M. (1972) The role of orientation constraints and rotational diffusion in bimolecular solution kinetics. J. Phys. Chem. 76, 534.

Schurr, J.M. (1976) Orientation constraints and rotational diffusion in bimolecular solution kinetics. A simplification. J. Phys. Chem. 80, 1934.

Silhavy, T.J., Szmelcman, S., Boos, W. and Schwartz, M. (1975) On the significance of the retention of ligand by protein. Proc. Natl. Acad. Sci. U.S.A. 72, 2120.

Stenberg, M., Elwing, H. and Nygren, H. (1982) Kinetics of reaction zone formation with radial diffusion of ligands over a receptor-coated surface. J. Theor. Biol. 98, 307.

Stenberg, M. and Nygren, H. (1982) A receptor-ligand reaction studied by a novel analytical tool-the isoscope ellipsometer. Anal. Biochem. 127, 183.

Stenberg, M. and Nygren, H. (1985) A diffusion limited reaction theory for a solid-phase immunoassay. J. Theor. Biol. 113, 589.

Stenberg, M., Stiblert, L. and Nygren, H. (1986) External diffusion in solid-phase immunoassays. J. Theor. Biol. 120, 129.

Stenberg, M., Werthén, M., Theander, S. and Nygren, H. (1988) A diffusion limited reaction theory for a microtiter plate assay. J. Immunol. Methods.

Steward, M.W. (1986) Overview: Introduction to methods used to study the affinity and kinetics of antibody-antigen reactions.. In: D.M. Weir, (Ed.), Handbook of Experimental Immunology, Vol. 1. Blackwell, Oxford, pp. 25.1–25.30.

Steward, M.W. and Lew, A.M. (1985) The importance of antibody affinity in the performance of immunoassays for antibody. J. Immunol. Methods 78, 173.

Trurnit, H. (1954) Studies on enzyme systems at a solid-liquid interface. II. The kinetics of adsorption and reaction. Arch. Biochem. 51, 176.

Uzgiris, E.E. and Kornberg, R.D. (1983) Two-dimensional crystallization technique for imaging macromolecules, with application to antigen-antibody-complement complexes. Nature 301, 125.

Wank, S.A., DeLisi, C. and Metzger, H. (1983) Analysis of the rate-limiting step in a ligand-cell receptor interaction: the immunoglobulin E system. Biochemistry 22, 954.