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Kinetics of antibody binding at solid-liquid interfaces in flow

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We have developed the theoretical framework for a displacement immunoassay conducted in flow under nonequilibrium conditions. Using a repetitive displacement technique, we determined the displacement rate and apparent dissociation rate constant at different flow rates. Our data suggest that the kinetics are best described by a first-order function. The displacement efficiency, the displacement rate, and therefore the apparent dissociation rate constant were calculated and demonstrated to be flow rate dependent. The theoretical framework developed in this study was successful in predicting the behavior of antigen displacement in flow.

Key words: Displacement; Immunoassay; Biosensor

Introduction

Immunoassays provide valuable tools for applications as diverse as clinical diagnostics, environmental monitoring, and process control. Based on the extreme specificity of antibody binding, immunoassay techniques are reliable and well-established. The development of solid-phase methods such as enzyme-linked immunosorbent assays (ELISA), in which the antibody or antigen is immobilized, has further extended the versatility of these assays by providing a simple means of separating bound and free reactants. As a result, ELISA-type assays are among the most widely used assays for medical diagnosis. However, due to multiple steps and time-consuming incubation periods, these methods are not suitable for fast analyses. A solid-phase immunoassay has been developed recently which operates in continuous flow and uses immobilized antibodies in an unconventional way (Kusterbeck et al., 1990; Bredehorst et al., 1991). This approach differs from other solid phase immunoassays by not requiring incubation and washing steps or the introduction of additional reagents following sample loading. Also in contrast to many existing approaches, this assay relies on the antigen/antibody dissociation event for the successful detection of the compound of interest.

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* *Present address:* Immunochemicals, Sigma Chemical Company, St. Louis, MO, USA. *Abbreviations:* ELISA, enzyme linked immunosorbent assays; IgG, immunoglobulin G; MASS, membrane affinity separation system; PBS, phosphate-buffered saline; BBS, borate-buffered saline.

In the flow immunoassay, immobilized antibody is first saturated with fluorophore-labeled antigen. The antigen-antibody mixture is then placed in a buffer flow. When unlabeled antigen is introduced, a proportional amount of fluorophore-labeled antigen is displaced from the binding sites of immobilized antibodies and subsequently detected downstream. Detection of picomole quantities of antigen occurs within seconds.

Designing a flow immunoassay with maximum detection sensitivity requires a detailed understanding of the displacement kinetics at the solid-liquid interface under flow conditions. At present, however, there is no theoretical framework for the optimal design of such assays. The kinetics of binding of antibody to antigen is different for immobilized antibody than for antibody in solution, and classical concepts for describing antibody-antigen interaction in fluid phase systems under equilibrium conditions do not adequately explain the continuous flow displacement reaction. Furthermore, the rapid response time of the flow immunoassay contradicts the binding kinetics of antigen (or antibody) to immobilized antibody (or antigen) as it is documented in ELISA-type assays. In the ELISA systems, performed under static conditions, several hours are required before measurable dissociation occurs (Nygren et al., 1987). The functionally irreversible nature of antigen/antibody binding at solid surfaces has been attributed primarily to the limitations imposed by the diffusion of dissociated reactants away from the surface (Stenberg and Nygren, 1985). In the flow system, as in ELISAs, the apparent irreversibility of antigen binding may be due primarily to the immediate reassociation of labeled antigen with the immobilized antibody. The unique feature of the flow immunoassay is that the bound labeled antigen can be rapidly displaced from the column following the introduction of free antigen.

In this study, we investigated the apparent discrepancy between antibody-antigen interaction at solid-liquid interfaces in ELISA-type systems versus the continuous flow system. Using a flow immunoassay designed for the detection of cocaine, a repetitive displacement technique was developed to determine the displacement kinetics under nonequilibrium flow conditions. The theoretical framework, formulated from the data of these experiments, was successfully applied to predict the performance of the immunoassay at different flow rates.

Materials and methods

Monoclonal antibody purification

Anti-benzoylecgonine monoclonal antibody specific for both cocaine and its major metabolite, benzoylecgonine, was purchased as ascites from Biodesign Corporation (Kennebunkport, ME). The IgG fraction was purified from the ascites using the membrane affinity separation system (MASS) (Nygene Corp., Yonkers, NY). Briefly, the 10 mg MASS device was used with a Pharmacia P-1 peristaltic pump (Piscataway, NJ), at a flow rate of 1.5 ml/min. Ascites (2 ml) was diluted to 50 ml with 0.15 M phosphate-buffered saline (PBS), pH 7.2. The MASS device was equilibrated with 50 ml of wash buffer before application of diluted ascites. After application of ascites, the MASS device was washed with PBS and the IgG-containing fractions of antibody were eluted from the membrane with 0.1 M glycine, pH 2.5. Antibody-containing fractions were immediately neutralized with borate-buffered saline (BBS), pH 9.0. Pooled antibody fractions were concentrated using 24 Centriflo ultra-filtration membrane cones (Amicon Corp., Danners, MA).

Antibody immobilization

Purified anti-benzoylecgonine antibody was coupled to tresyl chloride-activated Sepharose 4B (Pharmacia, Piscataway, NJ) at various concentrations using the following conditions. The required amount of tresyl chloride-activated Sepharose 4B gel was suspended in 1 mM HCl and washed for 1 h in 1 mM HCl. Approximately 200 ml of 1 mM HCl per gram of dried powder was used. Purified antibody was added to 5 ml of coupling buffer (0.1 M $NaHCO_3$ with 0.5 M NaCl) and incubated with the washed Sepharose in a stoppered vessel overnight at 4°C on a platform rocker. Excess antibody was washed away with coupling buffer and any remaining reactive groups were blocked with 0.1 M Tris-HCl, pH 8.0 for 4 h at 4°C. The

gel was washed with three cycles of alternating buffers. Each cycle consisted of a wash with 0.1 M acetate buffer, pH 4.0 containing 0.5 M NaCl and a wash with 0.1 M Tris buffer, pH 8.0 containing 0.5 M NaCl. The amount of antibody immobilized to the Sepharose 4B was quantified using the method of Ahmad and Saleemuddin (1985). The antibody-coupled Sepharose 4B gel was stored in PBS containing 0.02% sodium azide.

Synthesis of fluorophore-labeled antigen

A fluorescent label, fluorescein cadaverine from Molecular Probes (Eugene, OR), was coupled to the antigen, benzoylecgonine, using the following protocol. Benzoylecgonine hydrate, 4 mg (Sigma Chemical Co., St. Louis, MO) was dissolved in 3 ml of dioxane and allowed to cool on ice. Triethylamine (70 μl) and ethylchloroformate (53.5 mg) were added to the mixture and stirred on ice for 30 min. The fluorescein cadaverine, 9 mg, was added to 1 ml of dioxane with 20 μl of triethylamine. A few drops of distilled water were added to the mixture to help dissolve the fluorescein cadaverine. The fluorescein cadaverine mixture was added to the above benzoylecgonine solution and allowed to stir for 2 h at room temperature. The solution was dried, redissolved in 1 ml methanol, and purified using silica gel thin layer chromatography, Type PLK5F from Whatman (Maidstone, KY). The preparative plate was developed with methanol : chloroform (1 : 3). A single band at $R_f = 0.7$ was extracted with methanol. The final product was lyophilized and stored at 4°C. The concentration of fluorophore-labeled benzoylecgonine was calculated by comparison to a standard curve depicting the absorbance at 490 nm of a known concentration of fluorescein cadaverine.

Flow immunoassay

The flow immunoassay set-up consisted of a Jasco 821-FP fluorimeter (Easton, MD), a disposable microcolumn (Isolab, Akron, OH), a Hewlett Packard integrator (Palo Alto, CA) and a small peristaltic pump for buffer delivery. Aliquots of 50 mg drained antibody-coated Sepharose 4B were incubated 72 h at 4°C with a 100-fold molar excess of fluorophore-labeled antigen over total immobilized antibody. The entire mixture was added to a micro-column (5.5 cm × 0.5 cm) and washed with PBS until background fluorescence was less than 0.04 U. Column effluent was di-

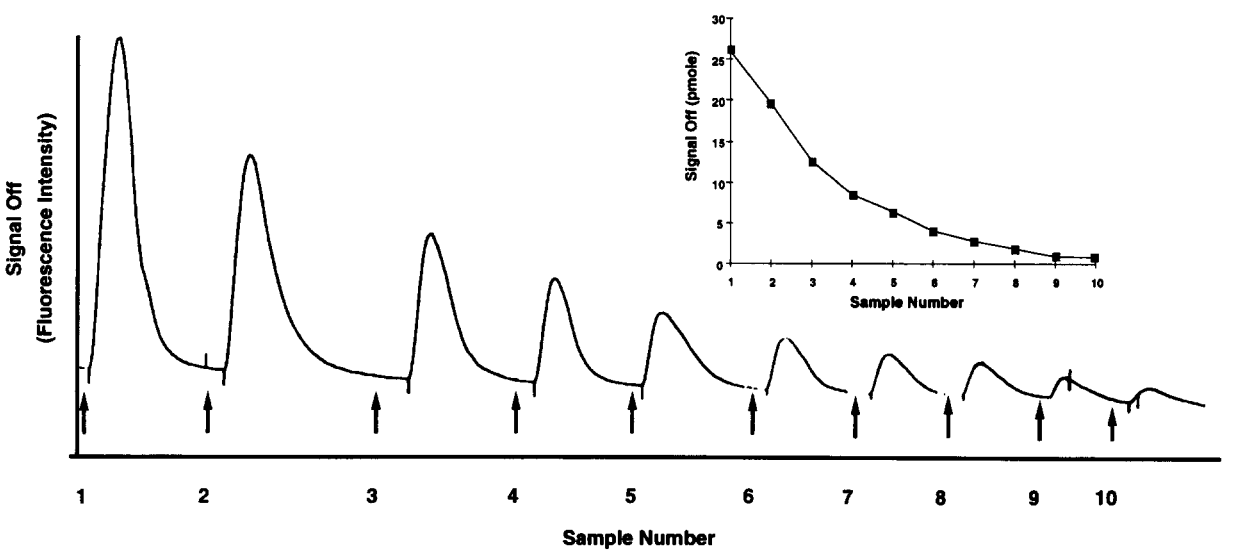

Fig. 1. Use of flow immunoassay to detect cocaine. Shown is the actual signal generated by the repetitive displacement of fluorescein-labeled benzoylecgonine. The inset plots the amount of fluorescein-labeled benzoylecgonine displaced as each sample was introduced. The amount was determined by comparing the integrated area of the fluorescence intensity to a standard curve. Duplicate runs illustrated standard deviations variations of ±3%.

rected to the fluorimeter through a flow cell, with fluorescence monitored at an excitation of 490 nm and emission of 520 nm. In a typical flow immunoassay, cocaine samples diluted in PBS were applied and delivered in continuous flow in a 200 μl volume. Following the introduction of unlabeled antigen, displacement of the fluorophore-labeled antigen was measured at various flow rates and antigen concentrations.

The kinetics of labeled antigen displacement were determined using a repetitive displacement technique. For these experiments, identical amounts of unlabeled cocaine were injected repeatedly until the column was depleted of fluorophore-labeled antigen. Using this procedure, the relatively low number of unoccupied antibody binding sites which may occur following the establishment of the buffer flow were filled upon the first injection of antigen and the contribution of these sites to the subsequent displacement data were minimized. The value for 'releasable' labeled antigen was determined by measuring the total amount of fluorophore-labeled cocaine which could be displaced during multiple additions of cocaine at a 100-fold molar excess.

Results

Flow immunoassay performance

Using the flow immunoassay, multiple samples could be run on a single column. Fig. 1 illustrates the displacement of labeled antigen by ten antigen samples at a constant flow rate of 1.0 ml/min

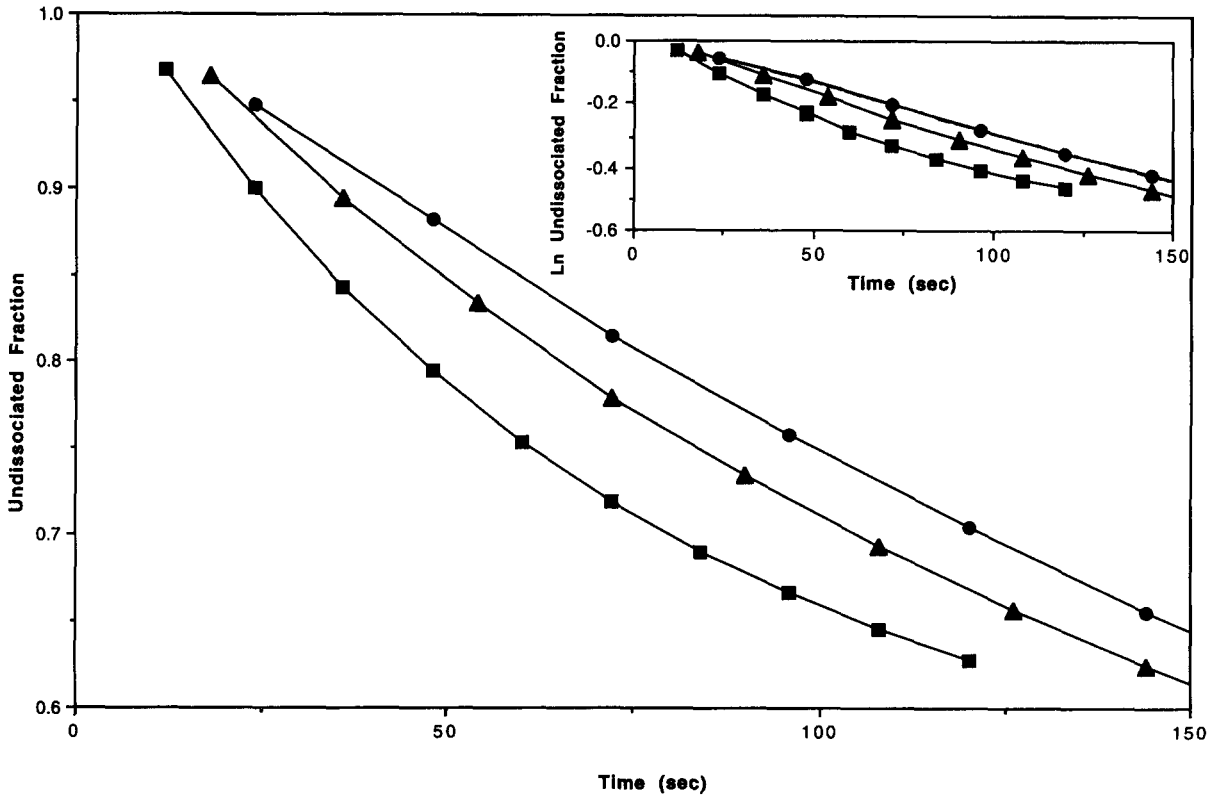

Fig. 2. Effect of flow rate on displacement. Continuous flow columns were created following the protocol outline in Fig. 1 at flow rates of 1.0 (■), 0.75 (▲), 0.5 (●) ml/min. The ratio of antibody to displaceable antigen was 2.6:1. After each application of cocaine, the undissociated fraction was plotted vs. the cumulative time of exposure of the column to cocaine. The data represent the mean of two experiments. The inset shows a linear relation when the natural logarithm (ln) of the undissociated fraction is plotted versus time, indicating a first order characteristic of the displacement rate. The coefficient of regression was 0.98, 0.99 and 1.0, at flow rates of 1.0, 0.75 and 0.5 ml/min, respectively.

and a ratio of unlabeled to releasable labeled antigen of 26:1. The releasable antigen, defined as [bound Ag*]$_{t=0}$, was determined by measuring the total amount of fluorescein-labeled benzoylecgonine displaced during multiple additions of cocaine at high excess (100-fold over available antibody-binding sites). By using this value as the total bound labeled antigen, any labeled antigen irreversibly bound to the column is irrelevant to both data analysis and to the function of the immunoassay. An exponential decrease was observed for the amount of labeled antigen released as a function of the number of injections (Fig. 1, inset), indicating a first order process.

Effect of flow rate on immunoassay

To investigate the effect of flow rate on the displacement kinetics, the data obtained from repetitive displacement experiments were used to calculate (a) the undissociated fraction of labeled antigen at each step of the repetitive displacement procedure and (b) the time of exposure of the antibody-bound labeled antigen to the unlabeled antigen, that is, the time allowed for displacement of labeled antigen. The undissociated fraction (θ) was calculated from the difference between total bound labeled antigen and the amount displaced after each addition of unlabeled antigen using the following relation:

$$\theta = \frac{[\text{bound Ag}^*]_{t=0} - [\text{displaced Ag}^*]_{\text{total}}}{[\text{bound Ag}^*]_{t=0}} \qquad (1)$$

where Ag* represents the amount of labeled antigen and the denominator represents the concentration of Ag* initially bound to the column (i.e., $t = 0$). Using this equation, θ was determined for each point during a repetitive displacement experiment at a constant flow rate.

To evaluate the effect of different flow rates, the same displacement experiment was repeated at flow rates of 0.5, 0.75, and 1.0 ml/min and the values for θ were calculated. The time period available for displacement was determined by dividing the volume of the column containing the immobilized antibody by the flow rate. Calculated values for the undissociated fraction were plotted as a function of displacement time (Fig. 2). Different slopes were observed for the various flow rates, reflecting differences in the rate of depletion of labeled antigen from the column (displacement rate). As depicted in the inset of Fig. 2, the function could be linearized by plotting the natural logarithm of the undissociated fraction as a function of the displacement time, thereby reconfirming the first order characteristic of the displacement kinetics.

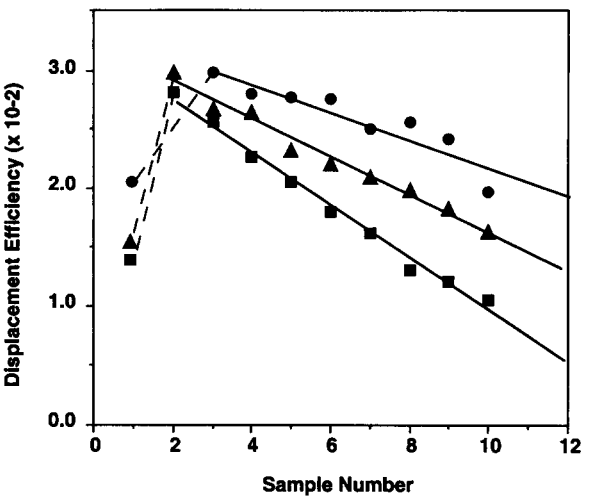

Fig. 3. Effect of flow rate on displacement efficiency. The displacement efficiency calculated using equation 2, was plotted versus the sample number for flow rates of 1.0 (■), 0.75 (▲), and 0.5 (●) ml/min. The values for D_e exhibited a first order relationship with curve fits of 0.9, 0.9 and 0.8, respectively.

Calculations of displacement efficiency and displacement rate

Taking this analysis further, the efficiency of each displacement event was determined. In the flow immunoassay, displacement efficiency, D_e, is described by the following relationship:

$$D_e = \frac{[\text{displaced Ag}^*]}{[\text{loaded Ag}]} \cdot \frac{1}{\theta} \qquad (2)$$

The displacement efficiency at flow rates of 0.5, 0.75, and 1.0 ml/min were then plotted as a function of the sample number (Fig. 3). Presumably, due to the availability of free antibody binding sites, the first injection in each experiment showed a lower integrated fluorescence intensity. Therefore, data obtained from the first injection were not considered in calculation of linearity.

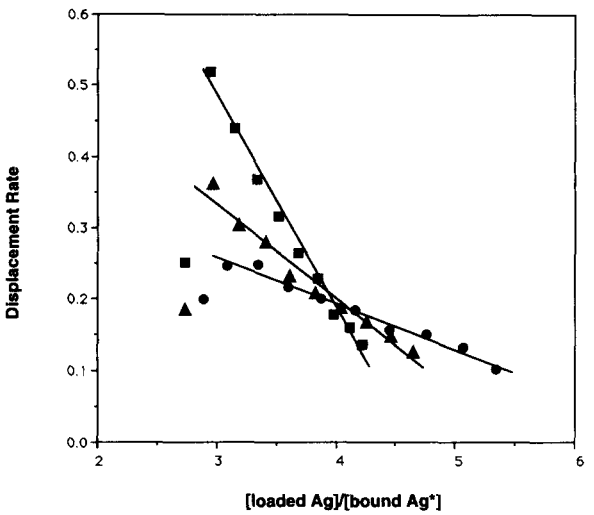

Fig. 4. Effect of flow rate on the rate of displacement. The rate of displacement of fluorophore-labeled antigen was calculated as in equation 3 and plotted as a function of the molar ratio of applied antigen to bound labeled antigen. Displacement rates (D_r) were calculated for flow rates of flow rates of 1.0 (■), 0.75 (▲) and 0.5 (●) ml/min.

The plot illustrates that the displacement efficiency increases with decreasing flow rates.

These experiments also permitted the determination of the rate of displacement of the labeled antigen from the antibody for the flow immunoassay. The rate of displacement (D_r) can be formally expressed as:

$$D_r = \frac{[\text{displaced Ag}^*]}{[\text{time}]} \tag{3}$$

where the denominator reflects the time period available for displacement. Displacement rates for the different flow rates of 0.5, 0.75, and 1.0 ml/min and plotted versus the molar ratio of unlabeled to bound labeled antigen. The maximum displacement rate occurs at the highest flow rate (Fig. 4), suggesting that reassociation of labeled antigen is minimized at the fastest flow rate.

Calculations of apparent dissociation constant

Equation (1) assumes that the dissociation consists of a first order process, and the data in Fig. 1 supports that assumption. To determine the apparent rate constant, the undissociated fraction, θ, can also be defined as a function of the dissociation time constant as follows:

$$\theta = e^{-t/\tau} \tag{4}$$

where t is the time available for displacement, and τ is the dissociation time constant. Only the

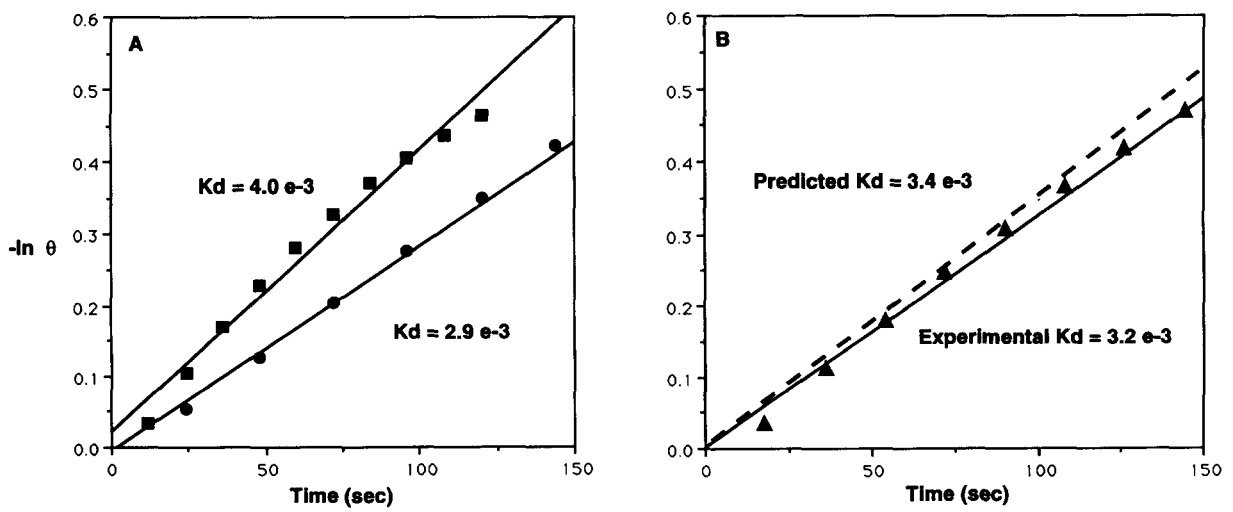

Fig. 5. Determination of apparent dissociation rate constants. In *A*, the negative of ln is plotted as a function of time for the flow rates of 1.0 (■) and 0.5 (●) ml/min. The slopes represent the dissociation rate constant (k_d). *B* shows the predicted (- - - - -) and actual (▲ ——— ▲) plots of the negative ln θ as a function of time for the flow rate of 0.75 ml/min.

behavior of the releasable fraction of labeled antigen is monitored. The apparent dissociation time constant can be determined by rewriting the equation as:

$$\tau = -\frac{t}{\ln \theta} \tag{5}$$

where the dissociation time constant τ is defined as the inverse of the first order apparent dissociation rate. The following equation describes the relationship between the apparent dissociation rate constant (k_d) and the undissociated fraction (θ):

$$k_d = -\frac{\ln \theta}{t} \tag{6}$$

Fig. 5 demonstrates that the apparent dissociation rate (k_d) is affected by the flow rate. To characterize the flow rate dependency, the k_d values obtained for flow rates of 0.5 and 1.0 ml/min were fitted to a first-order curve plot, as shown in Fig. 5A. To predict the apparent k_d for a flow rate of 0.75 ml/min, an average of the product of the slope and the flow rate for 0.5 and 1.0 ml/min was used. The values for θ determined from Fig. 2 were then used to calculate the actual apparent dissociation rate at 0.75 ml/min. As shown in Fig. 5B, the theoretical curve closely approximates the experimental values.

Discussion

Antibody-antigen binding in a homogeneous liquid phase is a well-studied process, and the kinetics of the reaction have been shown to follow the law of mass action (Karush, 1978; Kabat, 1980; Steward, 1986). The kinetics of antibody-antigen reactions observed in a flow immunoassay, however, do not reach the dynamic equilibrium as predicted from fluid-phase experiments (Kusterbeck et al., 1990). Based on the fluid phase dissociation constant ($k_d = 8.4\ s^{-1}$) of the monoclonal anti-dinitrophenol used in the experiments of that study, more than 99.9% of the antibody-bound antigen of the flow immunoassay column should be washed away within 5 min of establishing the buffer flow. The experiments revealed, however, that the amount of antibody-bound antigen decreased slowly, despite extensive washing. Identical results were obtained in the experiments of the present study using an anti-benzoylecgonine antibody, indicating stable binding of labeled antigen to the immobilized antibodies of the flow immunoassay column. In the presence of competing unlabeled antigen, however, displacement of labeled antigen from the antibody binding sites occurs within seconds, as demonstrated by the short period of time required for the detection of released labeled antigen molecules.

For other assays performed on solid surfaces under static conditions, antibody binding proved to be so stable that the antibody-antigen reactions have been considered virtually irreversible (Mason and Williams, 1980). The mechanism responsible for the apparently irreversible antibody binding at solid-liquid interfaces has been suggested to be mass transport limitation (Berzofsky and Berkower, 1984; Stenberg and Nygren, 1988). Coated microwells routinely used in ELISA-type assays, with high local densities of antibody or antigen, favor rapid reassociation instead of diffusion and transport away from the surface following dissociation. In one study, even in the presence of large excess of soluble antigen, the dissociation of antibodies from surface-immobilized antigen was found to be negligible over a period of approximately 3 days (Nygren, et al., 1985).

It is obvious from these data that the theoretical framework developed for static ELISA systems cannot be applied to describe the kinetics of antibody-antigen interactions occurring in a continous flow immunoassay. Several parameters differ significantly. First, buffer flow reduces the limitations of diffusion as observed in static ELISA systems. Second, the association rate of antigen with immobilized antibody has been reported to decrease with increasing flow rates (Lin et al., 1989). Furthermore, the surface density of immobilized antibodies in the flow immunoassay is at least 2–3 orders of magnitude lower than in ELISA systems. Assuming the smallest possible surface area of the solid support in the flow immunoassay columns, that is spherical Sepharose

beads with no available internal surface area, the surface density of immobilized antibody is maximally 3×10^{-14} mol/cm^2. This value is two orders of magnitude lower than the surface density of antibodies immobilized onto microwells (1.5×10^{-12} mol/cm^2) (Nygren et al., 1987). The lower surface density of immobilized antibody can be expected to significantly limit the probability of reassociation events.

In the present study, we used a repetitive displacement technique to characterize the flow immunoassay by determining the displacement rate and apparent dissociation constants k_d at different flow rates. The data revealed several important characteristics of antibody-antigen interactions at solid-liquid interfaces under the non-equilibruim conditions of the flow immunosensor. First, the displacement kinetics are best described by a first order function. Second, the displacement efficiency, the displacement rate, and therefore the apparent dissociation rate constant, are flow-rate dependent. The maximal displacement *efficiency* was observed at the lowest flow rate, whereas the the maximal displacment *rate* occurred at the highest flow rate. The apparent dissociation rate also increased with increasing flow rates. It has been reported that increasing the flow rate decreases the association rate of antigen with immobilized antibody (Sportsman and Wilson, 1980). This paper demonstrates that not only the association rate but also the dissociation rate of immobilized antibody-antigen complexes is flow-rate dependent.

Using the theoretical framework developed, we can predict the behavior of antigen displacement events in the flow immunoassay described here. As shown in this study, theoretically calculated and experimentally determined apparent dissociation rates were very close. We are aware that a number of additional parameters which influence the performance of the flow immunoassay, including antibody heterogeneity due to random immobilization, antibody affinity, solid phase effects, and differential affinity for labeled versus unlabeled antigen, were not considered. However, the availability of the model presented in this paper will, in the future, permit the incorporation of these additional variables known to influence immunoassays.

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