Kinetic and Equilibrium Studies of Insulin Immunoaffinity Chromatography

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The kinetic and equilibrium characteristics of the interaction of insulin with a specific antibody immobilized on silane-treated controlled pore glass beads have been evaluated by using the technique of "high-performance immunoaffinity chromatography" (HPIC). A previously described equilibrium model was verified and used to establish the equilibrium constants for the reaction of the immobilized antibody with insulin in the mobile phase. Computer simulations and experimental data were used to demonstrate the contribution of nonspecific interactions with the support to the overall binding process. An estimate is also made of the rate of the antibody–antigen complex formation reaction, and its effect on immunochromatographic investigations is discussed.

Immunoaffinity chromatography is a widely used technique for the purification of antibodies and antigens (*1*). A column consisting of covalently immobilized antibody or antigen ("immunosorbent") allows the convenient and selective sorption of the soluble component (ligate). It specifically binds to the immunosorbent from a mixture of contaminants which are washed away prior to recovery of the ligate through desorption by some change in mobile phase composition, typically a change in pH (*1*). In a previous report (*2*) we demonstrated that antibodies immobilized on silane-treated porous glass could be used as immunosorbents at relatively high flow rates. Thus we designated the technique high-performance immunoaffinity chromatography (HPIC). The application of HPIC to rapid immunoassays and protein purification requires that limitations imposed by the kinetics and thermodynamics of interphase interactions be assessed. It has been shown that the limit of detection in radioimmunoassays is on the order of $0.1K_0^{-1}$ where K_0 is the average affinity constant of the antiserum (*3*, *4*). By similar arguments it can be shown that the performance of purification schemes based on immunoaffinity chromatography will depend on the relative magnitudes of the affinities and concentrations of the substances involved. The kinetics of these systems will also be important to optimization of experimental conditions.

It was shown previously that the binding of antigen by an HPIC column could be described in terms of an apparent binding constant, K', and an apparent total binding site concentration $(Ab)_t$ whose values could be determined by assessing the extent of antigen binding as a function of concentration according to quasi-equilibrium arguments (*2*). The values of K' were shown to vary inversely with flow rate.

Given these observations, we decided to investigate whether an increase in concentration of antibody binding sites would show up as a corresponding increase in the value of $(Ab)_t$ and whether this would affect the value of K'. Since the value of K' appears to change with flow rate and hence residence time of antigen in the column, we also undertook kinetic studies to further elucidate this phenomenon.

We report results here which demonstrate the kind of behavior predictable by such an equilibrium treatment. For this study we have elected to use the binding of the antigen insulin by an antiserum exhibiting high affinity in order to be able to investigate the behavior of the system down to subnanomolar antigen concentrations.

EXPERIMENTAL SECTION

Materials. Lichrospher SI 1000 (10 μm diameter microporous silica of 100 Å pore size) was obtained from MC/B Manufacturing Chemists, Inc., Cincinnati, OH. Glycidoxypropyltrimethoxysilane (GOPS) was obtained from Petrarch Systems, Inc., Levittown, PA. Crystalline bovine zinc insulin was the gift of Mary Root of Lilly Research Laboratories, Indianapolis, IN. [^{125}I]insulin (porcine), 130 μCi/μg, was obtained from New England Nuclear, Inc., Boston, MA, and was purified by gel filtration on Sephadex G-75F (Pharmacia, Inc., Piscataway, NJ) (50 cm × 1 cm), using the second hot peak material (*5*) which comprised antigen of high (>90%) immunoreactivity. Competitive binding studies of this material with subnanomolar concentrations of unlabeled insulin showed that labeling did not significantly affect binding characteristics and demonstrated the similar high affinity of the antiserum for both porcine and bovine insulin.

Lyophilized antisera to porcine insulin, raised in the capybara, were the gift of Ernest C. Adams of Miles Laboratories, Inc., Elkhart, IN, and were received as lyophilized whole sera (*5*). The globulin fraction of the reconstituted antisera was prepared by precipitation with Na_2SO_4 three times, followed by dialysis against 0.2 M borate buffered saline, 0.85%, pH 9.0 (BBS). The specific antibody was shown to comprise 7% of the total globulin protein by a Scatchard plot of binding data obtained originally in the form of a competitive binding assay standard curve for insulin in the region of nanomolar concentrations. The hyperbolic shape of the Scatchard plot suggested that the native antiserum exhibited affinities for the antigen in the range of 10^7–10^{10} L/mol. Bovine serum albumin (BSA), "RIA grade", was obtained from Sigma Chemical Co., St. Louis, MO. Normal guinea pig serum gamma globulin, purified by preparative isoelectric focusing, was the gift of Perry Cole of the V.A. Hospital, Tucson, AZ. This was used as a control for nonimmunospecific binding since normal capybara globulin was not available. The close immunochemical similarity of capybara and guinea pig gamma globulin has been reported (*5*).

The silanization of the silica support by aqueous GOPS to give a diol bonded phase was performed as previously described (*2*). This bonded silica was given the designation "G23".

The capybara immune globulin or normal guinea pig gamma globulin (NGPG) was then attached to this support by reductive alkylation as also described in ref 2. Analysis of the resulting capybara globulin immunosorbent by hydrolysis in 6 N HCl (*2*) followed by Folin-Lowry protein assay (*6*) gave a value of 6.3 mg of protein/g of glass (dry weight basis). The antiserum as supplied was labeled "CAPY. 2". The resulting immunosorbent was given the abbreviated designation "CP2-G23".

Apparatus. Spectral measurements were made with a Cary 219 UV-VIS spectrophotometer. Counting of [^{125}I]insulin was done on a Nuclear Chicago Model 4454 gamma well counter. The liquid chromatograph used was a SpectraPhysics Model 8700 solvent delivery system. The column used for HPIC work is shown in Figure 1. It consists of a Swagelok ZDV $^1/_4$ in. to $^1/_{16}$ in. reducing union with a 2-μm stainless steel frit secured into the bottom. The actual "column" consists of 50–70 mg of derivatized silica support material. A silanized glass wool plug takes up any dead space between the column head and the special inlet fitting, which is made by silver-soldering a $^1/_{16}$ in. (0.002 in. i.d.) 316 stainless steel (SS) tube into a $^1/_4$ in. i.d. (0.2 cm i.d.) 316 SS sleeve

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Table I. Effect of Immunosorbent Concentration on Observed $[Ab]_t$ and K' as Determined by HPIC[a]

immunosorbent added, mg	added $[Ab]_t$,[b] M	obsd $[Ab]_t$,[c] M	% active[d]	K_L',[e] M^{-1}	$(B/F)_0$[f]
1.8	3.8×10^{-7}	4.9×10^{-9}	12.9	1.1×10^{7}	0.51
8.8	1.9×10^{-6}	1.5×10^{-7}	7.9	2.1×10^{7}	3.6
23.0	4.9×10^{-6}	4.3×10^{-7}	8.8	3.6×10^{6}	6.9
58	1.2×10^{-5}	1.1×10^{-6}	9.2	7.5×10^{5}	7.1
0[g]					0.17

[a] Conditions: 0.4 mL/min flow rate; antigen, insulin with [^{125}I]insulin as tracer in 0.2% BSA/PB; volume injected, 0.050 mL; elution scheme, see text. [b] Determined from volume of immunosorbent suspension added to suffcient diol bonded silica to make 70 mg of mixture and from known protein content of immunosorbent. [c] Determined from extrapolation of Scatchard plots. [d] Ratio of experimental $[Ab]_t$ to added $[Ab]_t$. [e] Determined from the limiting slope of line from Scatchard plot used to calculate $[Ab]_t$. [f] B/F ratio at zero unlabeled antigen concentration. [g] Column consists only of diol bonded phase.

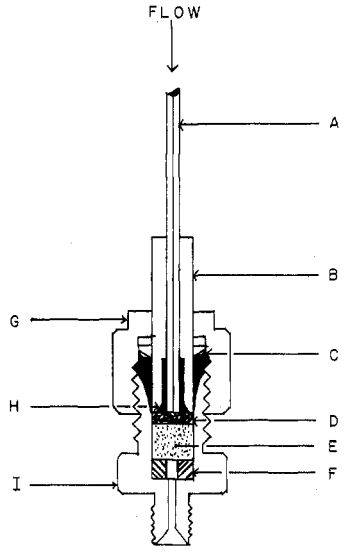

Figure 1. Column for HPIC studies: (A) $^1/_{16}$ in. o.d., 0.002 in. i.d., 316 stainless steel (SS) tubing; (B) $^1/_4$ in., 2.2. mm i.d. SS tubing; (C) $^1/_4$ in. ferrules; (D) glass wool plug; (E) immunosorbent; (F) 5-μm frit; (G) $^1/_4$ in. Swagelok nut; (H) silver solder seal; (I) ZDV reducing union.

as shown. Thus the column dimensions are 6 mm i.d. × 0.5 cm length. It was found that such columns are best packed by slurrying the immunosorbent in 0.1 M phosphate buffer with 0.8% NaCl (PBS) and allowing the particles to settle by gravity.

In the quantitative study of the effect of variation of antibody binding sites in HPIC, a series of columns as described above were packed with sorbents consisting of various microliter volumes of immunosorbent suspension (100 mg/mL) added to 70 mg of diol bonded phase. The mixture was ultrasonicated and then packed as described.

Binding measurements by HPIC were performed by making appropriate dilutions of antigen with 0.1% (w/v) BSA in PBS, adding [^{125}I]insulin to give an activity of 10 000 counts/min per 100 μL, and then injecting 50-μL samples onto the column at a given flow rate. At this point, the mobile phase is 0.1 M phosphate, pH 7.0. After 2 mL, including the "free" fraction (F), had been collected at the column outlet, the mobile phase is changed to 0.1 M sulfamic acid, pH 1.2, whereupon antigen bound to the antibody on the column is displaced (*2*). Eluting antigen is again collected as a 2-mL fraction (fraction B for "bound"). The ^{125}I activity of each fraction is then determined. Recoveries of activity were always above 95%. The temperature of the column was maintained at 33 °C by a thermostated water bath.

Binding data were evaluated as described in ref 2. It was shown previously that an apparent binding constant could be calculated by determining the ratio of bound to free antigen (B/F) and plotting this value against the concentration of bound antigen, for several initial concentrations of antigen (*2*). The method for determining equilibrum constants as given by Scatchard (*7*) is rearranged to give

$$B/F = [Ab]_t K' - [B]K' \quad (1)$$

Thus K' is determined from the slope of the plot, and $[Ab]_t$, the total binding site concentration, is given by the intercept on the abcissa. Since only the labeled antigen is directly measured, it is necessary to assume essential identity of labeled and unlabeled antigen, as in ordinary cold competition binding assays (*8*).

Method for HPIC Kinetic Measurements. The kinetics of insulin HPIC were studied at 33 °C by measuring the extent of binding of G-75 fractionated [^{125}I]insulin, the concentration of which was 0.05 ng/mL as calculated from the specific activity (130 μCi/μg) after allowing for isotopic decay time and counter efficiency. Aliquots of 50 μL were injected and "bound" and "free" fractions were collected as before.

Batch Kinetics. The kinetics of a batch mode binding of [^{125}I]insulin to immunosorbent were studied at 33 °C by adding 0.15 mL of [^{125}I]insulin (approximately 30 000 counts/min or 0.05 ng/mL) to 0.25 mL of a suspension of CP2-G23 in 1.5-mL microcentrifuge tubes (precoated with 1% BSA for 2 h, followed by rinsing and drying). The CP2-G23 suspension had 1.0 mg of immunosorbent/mL of BSA buffer.

The mixture was agitated at once and continuously until the desired time elapsed, at which point the tube was centrifuged at 12000*g* for 3 min, effectively stopping the reaction. The supernatants were decanted and the pellets counted.

RESULTS AND DISCUSSION

According to the previously developed model it might be predicted that increasing the antibody content of the HPIC column would affect only the parameter $[Ab]_t$ (eq 1), while the slope of the Scatchard plots would remain the same.

The results of varying the specific immunosorbent concentration of an HPIC column are summarized in Table I. The values of the parameter $[Ab]_t$ were determined by extrapolation of the Scatchard plots to the abscissa as in ref 2. A linear correlation exists between the weight of immunosorbent added and the value of $[Ab]_t$ subsequently observed in accordance with expected behavior.

The lower limiting slope of the extrapolated line in the Scatchard plot should be a measure of the lower limit of apparent binding constant K_L' for an immunosorbent exhibiting heterogeneity of binding affinities (*9*). In Table I it may be seen that these limiting affinities appear to decrease with increasing immunosorbent concentration.

The fact that the values of $[Ab]_t$ are ten-fold less than the amount of globulin protein known to be immobilized on the immunosorbent could be taken to support earlier conclusions that the immoblization of an antibody results in significant loss of activity (*2*, *10*). However, antibody comprised only about 7% of the total protein in the immune serum globulin. Thus when the expected 80%–90% loss of activity upon immobilization is taken into account, a value for the percent activity of less than 1.5 should be obtained instead of the higher values shown in Table I. This led us to suspect that the matrix of the immunosorbent might be contributing to the binding of the antigen through nonspecific interactions. Batch experiments run with unmodified diol bonded phase

Table II. Simulation of Equilibrium Binding of Antigen by a Support Material Exhibiting 3-Fold Heterogeneity[a]

simulation	$[Ab]_t$ actual, M	$[Ab]_t$ apparent, M	K_L', M^{-1}
1	2×10^{-9}	3.3×10^{-8}	3.5×10^{7}
2	2×10^{-8}	2.5×10^{-7}	5.6×10^{6}
3	1×10^{-7}	2.2×10^{-6}	9.3×10^{6}
4	2×10^{-7}	9.6×10^{-6}	6.4×10^{5}

[a] A support with components B1, B2, and B3 having equilibrium constants K_1, K_2, and K_3 reacts to equilibrium with monovalent antigen: $K_1 = 10^{10}$, $K_2 = 10^{8}$, and $K_3 = 10^{2}$ L/mol; $[B3] = 1 \times 10^{-3}$ M; $[B1] = [B2]$ and $[B1] + [B2] = [Ab]_t$ actual in all simulations. In all cases the range of antigen concentration [Ag] is from $5([B1] + [B2]) > [Ag] > 0.05([B1] + [B2])$.

and with immobilized NGPG showed a binding affinity for insulin of 10^2 to 10^4, indicating that nonspecific adsorption of the antigen is a significant process.

Computer simulation of the equilibrium binding of an antigen to a heterogeneous antibody was then undertaken to see whether the discrepancy of values for $[Ab]_t$ in Table I might be explained by invoking the presence of low affinity nonspecific interactions. Scatchard plots for hypothetical antigen–antibody binding experiments were generated. Here, antibody heterogeneity was mimicked by assuming the existence of a mixture of two discrete antibody populatons, each with its own affinity constant and concentration. The antibodies were assumed to be of two discrete types, having binding constants K_1 and K_2 of 1×10^{10} and 1×10^{8} L/mol, respectively. Their concentrations B1 and B2 were assumed to be equal. The third interaction was then assumed to involve sites on a diol bonded phase with a binding constant K_3 of 1×10^{2} and a concentration B3 equal to that of the diol moieties on the bonded phase (viz., approximately 100 μmol/g). This translates into an effective column concentration of diol of about 1×10^{-3} M. The monovalent antigen (Ag) was then assumed to react to equilibrium with all binding sites. Such a model can be represented by the relationship

$$[Ag]_b = \frac{[Ag]_f[B1]K_1}{[Ag]_fK_1 + 1} + \frac{[Ag]_f[B2]K_2}{[Ag]_fK_2 + 1} + \frac{[Ag]_f[B3]K_3}{[Ag]_fK_3 + 1} \quad (2)$$

where $[Ag]_b$ and $[Ag]_f$ represent bound and unbound antigen concentrations, respectively, at equilibrium. Concentrations of the antigen and antibodies in the simulated experiments were chosen to cover the ranges studied in the actual experiments. The results of several simulations are reported in Table II.

As can be seen, the presence of a high concentration of low affinity sites amidst relatively low concentrations of high affinity sites gives binding behavior, based on equilibrium models, which leads to an estimation of bonding site concentrations which is higher than that due solely to the high affinity sites. It can also be seen that the magnitude of this overestimation decreases as the high affinity site concentrations increase. Finally, the limiting slope of the Scatchard plots also decreases with an increase in the high affinity site concentration.

Precisely these trends, with similar magnitudes, are observed in the data of Table I. Figure 2 gives a comparison of theoretical and experimental results. As the concentration of the high affinity immunospecific sites ($[Ab]_t$) increases with respect to those of low affinity, the concentration range of antigen assessed must also be increased. A decrease in the limiting affinity, K_L', results, a trend which is seen both in experimental results and in the simulations; this is attributed to the increasing contributions to the overall binding from nonspecific sites at increasing Ag concentrations. The influ-

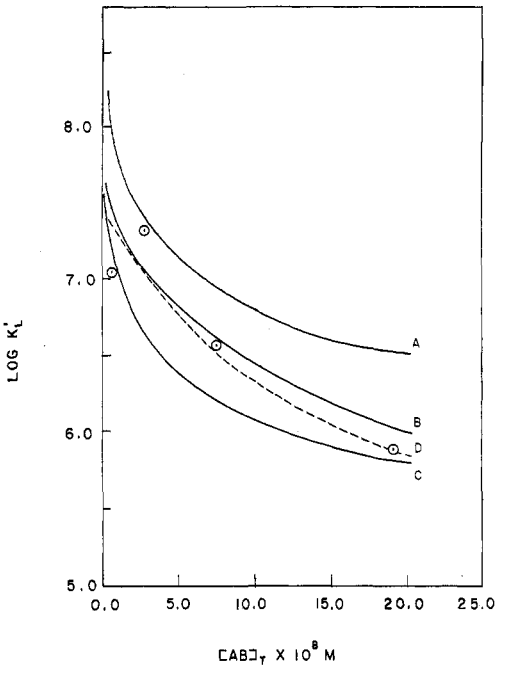

Figure 2. Effect of nonspecific binding on apparent affinity constants: (A) $K_1 = 10^{11}$, $K_2 = 10^{9}$ L/mol, $[B] = 1 \times 10^{-3}$ M; (B) $K_1 = 10^{11}$, $K_2 = 10^{9}$ L/mol, $[B3] = 2 \times 10^{-3}$ M; (C) $K_1 = 10^{10}$, $K_2 = 10^{8}$ L/mol, $[B3] = 1 \times 10^{-3}$ M; (D) line drawn through experimental data (circled points). Other conditions are as given in Table II.

ence of the low affinity sites is markedly illustrated by comparing curves A and B of Figure 2. Here, a doubling of the concentration of low affinity sites causes the theoretical curve to shift downward as shown; this is to be compared with the shift (curve C) caused by a 10-fold reduction in the antibody binding constants.

From a comparison of experimental and theoretical results we conclude that the change in K_L' seen in Table I is one predictable by equilibrium arguments. Furthermore, the assumptions made regarding the relative values of the various binding parameters are qualitatively consistent with the chosen model. It is also predicted from this model that the error in assessing the value of $[Ab]_t$ from equilibrium binding data is a function of both the concentration [B3] and affinity K_3 of the nonspecific site. Figure 3 shows that as the product of these two values increases relative to those of the high affinity interactions $[Ab]_t \times K_0$, where $\log K_0 = (\log K_1 + \log K_2)/2$, a point is reached after which the error in $[Ab]_t$ becomes intolerable. Using the approximate values of the parameters for our experimental data, we calculate an overestimation of about 10% in $\log [Ab]_t$, which agrees with the experimental error observed.

It seems reasonable therefore to assume that a quasi-equilibrium model for HPIC provides a simple but effective conceptual basis for understanding the individual contributions of matrix and antibody to the observed binding of antigen. It should be noted that these errors in estimation of binding parameters would not be expected to show up as concomitant errors in an immunoassay based on HPIC, since a calibration curve may still be generated with appropriate standards. However, when the extent of nonspecific binding (e.g., $[B3]K_3$) becomes large enough relative to specific binding ($[Ab]_tK_0$), the precision or steepness of the standard curve will be adversely affected and the accuracy will become sensitive to interferences from the analyte matrix.

That genuine biospecific interactions predominate in the present study is indicated by the low value of $(B/F)_0$, the B/F ratio of labeled antigen alone, when only diol bonded phase is present, as shown in Table I. Further, since these values

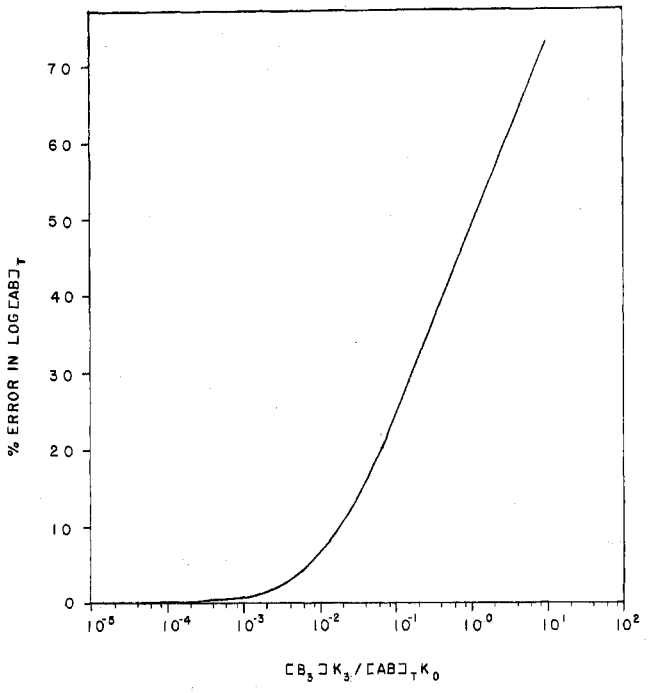

Figure 3. Effect of nonpspecific binding on determination of antibody concentration: $[Ab]_t = 2 \times 10^{-8}$ M; $K_0 = 1 \times 10^9$ M^{-1}.

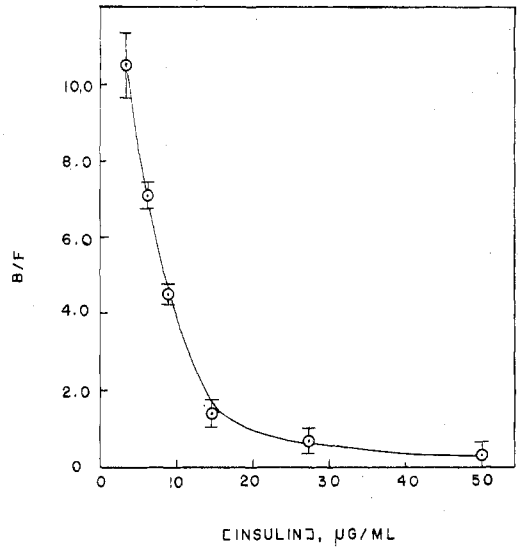

Figure 4. Standard curve for insulin immunoassay by HPIC: column, 5 mm × 6 mm i.d., CP2-G23 mixture (see text for composition); flow rate, 0.5 mL/min; volume injected, 50 μL; T, 33 °C. Bound to free ratio (B/F) is based on activity of tracer [^{125}I]insulin recovered in each fraction. Points shown represent average of duplicate determinations.

are obtained at the concentration of labeled antigen which is roughly equal to 1×10^{-11} M, the ability of the HPIC technique to perform extremely sensitive assays is indicated.

Figures 4 and 5 are standard curves for an insulin immunoassay at two different ranges of antigen concentration. In Figure 4, the standard curve covers the range of 0.1–2 μg/mL, and here the HPIC column is packed with 75 mg of immunosorbent only. In Figure 5, the standard curve covers the range 10–300 ng/mL, and the column has been packed with a mixture of 1 mg of immunosorbent and 70 mg of diol bonded phase.

Kinetic Studies. The approximation of equilibrium implies that the kinetics of HPIC are rapid. The range of flow rates over which analytical measurements may be made will depend on these kinetics as well. The rate of formation of the antibody–antigen complex in HPIC may be estimated by

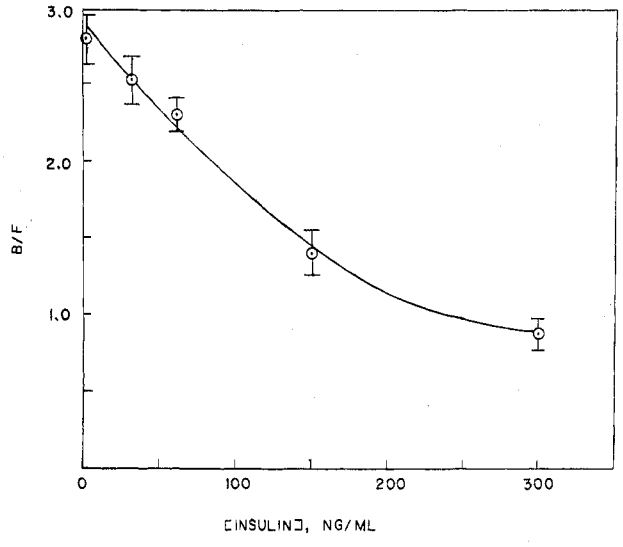

Figure 5. Standard curve for insulin immunoassay by HPIC. Conditions as in Figure 4. Column: see text for composition.

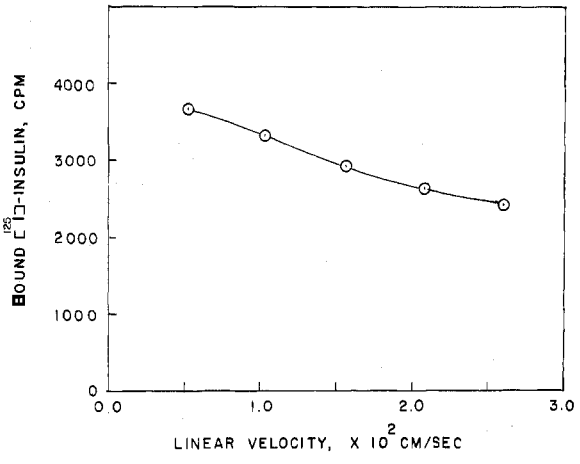

Figure 6. Flow rate dependence for the binding of [^{125}I]insulin to HPIC column. Column and other conditions are as given in Figure 4. Flow rates = 0.1–0.5 mL/min.

varying the flow rate and hence residence time of the antigen in the column.

Figure 6 is a plot of the flow rate dependence of the extent of [^{125}I]insulin binding to an HPIC column. It can be seen that a 5-fold decrease in flow rate results in only a 50% increase in the total number of counts bound; this would seem to indicate that at least two kinetically distinct binding processes are occurring: one fast, possibly diffusion limited, the other considerably slower. Assuming reversible second-order kinetics, one can treat the data of Figure 6 to obtain an apparent forward rate constant, k_f', for this slower process of 3.6×10^6 M^{-1} s^{-1}. This is to be compared with the results of the batch kinetics experiment whose results are shown graphically in Figure 7. In both cases the data are plotted according to the integrated form of a second-order reversible rate expression

$$f(X) = \frac{1}{(\bar{X}' - \bar{X})} \ln\left(\left|\frac{\bar{X}}{\bar{X}'}\right| \cdot \left|\frac{\bar{X}' - X}{\bar{X} - X}\right|\right) = k_f' t \qquad (3)$$

where $\bar{X}$ is the equilibrium value of bound antigen concentraton and X is the value at any time T; $\bar{X}'$ is given by

$$\bar{X}' = \frac{K([Ag]_0 + [Ag]_0) + 1}{K} - \bar{X} \qquad (4)$$

where $[Ag]_0$ and $[Ab]_0$ are initial antigen and antibody concentrations, respectively (*11*).

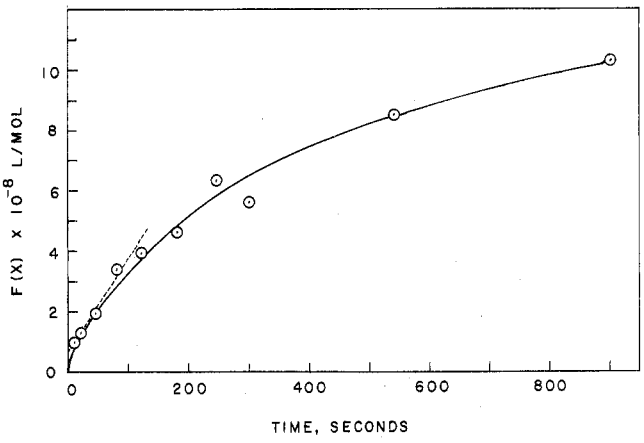

Figure 7. Batch mode kinetics of [^{125}I]insulin binding to CP2-G23 immunosorbent. Dashed line is the initial slope referred to in text.

It can be seen that the slope of this plot is clearly not a straight line. Since the kinetics of antibody–antigen reactions in both heterogeneous and homogeneous reaction phases are known to follow a second-order rate law (*12*, *13*) the curvature of this plot evidently is due to heterogeneity of antigenic binding sites and reflects the contribution of several separate forward rates (*14*) some of which may be due to nonspecific binding. Taking the initial slope ($10 \text{ s} < t < 120 \text{ s}$), a value for k_f' of $3.4 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ is obtained, comparable to the value obtained for the "slower" process alluded to above in the description of flow rate dependence of antigen binding.

The nonzero intercept of this line suggests that still faster reactions (greater than $10^7 \text{ M}^{-1} \text{ s}^{-1}$) are occurring which cannot be measured by present techniques. Studies at higher flow rates were then undertaken to observe these faster kinetics. The results of such studies at 0.5–3.0 mL/min gave a linear relationship when plotted according to eq 2 and a forward rate of $3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$. It has been noted that the forward step of many antigen– and hapten–antibody reactions proceeds at diffusion-limited rates (*7*). The observed "fast" kinetics are consistent with such processes.

Affinity chromatography has been proposed as a method for the determination of rapid forward rate constants on the basis of a statistical treatment of band broadening of an isocratically eluted peak (*15*). Such a model assumes relatively low affinity and fast reverse rate constants. As we have shown here, an apparent forward rate constant k_f' of $3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ is obtained by kinetic assessment of the flow rate dependence of antigen binding. If the average affinity K_0 of the immunosorbent is conservatively estimated to be $5 \times 10^9 \text{ M}^{-1}$, a value of $6 \times 10^{-3} \text{ s}^{-1}$ is obtained for k_r', the apparent reverse rate constant. On the time scale of these experiments, an essentially irreversible reaction thus results, and the error due to dissociation of specific complexes can be considered negligible. Indeed, the irreversible nature of insulin binding to high affinity antisera has already been reported (*16*). We have observed that the recovery of bound [^{125}I]insulin is not measurably affected by long (10 min) delays between adsorption and desorption, consistent with the above calculations.

In this study it has been assumed that the rate-limiting step in binding is the intrinsic forward rate of the antibody–antigen reaction, and that the effect of changing the flow rate is merely one of changing the residence or reaction time of the antigen in the column. Horvath has demonstrated that the major factor leading to nonequilibrium in high-performance liquid chromatography (HPLC) is the kinetic limitation of solute–stationary phase interaction and not mass transport phenomena, provided that particle size and flow rates are of magnitudes similar to those employed in this work (*17*). High affinity interactions with fast forward rate kinetics should show behavior approximating equilibrium at diminishing flow rates, an observation which we have noted previously (*2*).

We have observed in the course of this work that the nonspecific interactions of insulin with immunosorbents and unmodified silica proceed at much lower rates than those for the specific binding by immobilized antibody. This suggests that HPIC may be advantageously used in an isokinetic fashion to discriminate between biospecific and nonspecific binding on the basis of the slower rates of the latter. The ability to determine relatively rapid kinetics of antibody–antigen reactions by HPIC is indicated. This should prove useful in many areas of immunochemical research.

In conclusion it can be said that the theory of HPIC provides a model for assessing the effects of changes in binding site concentration. That this theory holds for systems utilizing such low concentrations as reported here bodes well for the development of rapid protein-binding assays by HPIC. The data shown here demonstrate that equilibrium is very nearly approached in HPIC as in other forms of chromatography and hence a quasi-equilibrium theory is appropriate and adequate to describe the binding behavior within the limits prescribed by the extent and nature of any competing nonspecific adsorptive processes.

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LITERATURE CITED

(1) Ruoslahtl, E., Ed. "Immunoadsorbents In Protein Purification"; University Park Press: Baltimore, MD, 1976.
(2) Sportsman, J. R.; Wilson, G. S. *Anal. Chem.* **1980**, *52*, 2013–2018.
(3) Yalow, R. S.; Berson, S. A. In "Principles of Competitive Protein Binding Assays"; Odell, W. D., Daughaday, W. H., Eds.; Lippincott: Philadelphia, PA 1977; p 1.
(4) Schuurmann, H. J; de Liguy, C. L. *Anal. Chem.* **1979**, *51*, 2–7.
(5) Adams, E. C.; Layman, K. M.; Hartnagel, R. *Ann. Clin. Lab. Sci.* **1974**, *4*, 19–28.
(6) Peterson, G. L. *Anal. Biochem.* **1977**, *83*, 346–356.
(7) Richard, R. F.; Rosenstein, R. W.; Varga, J. M.; Konigsberg, W. H. In "Immunoglobulins"; Litman, G. W., Good, R. A., Eds.; Plenum: New York, 1978; pp 138–141.
(8) Farr, R. S. *J. Infect. Dis.* **1958**, *103*, 239.
(9) Zettner, A. *Clin. Chem. (Winston-Salem, N.C.)* **1973**, *19*, 699–705.
(10) Line, W. E.; Siegel, S. J.; Kwong, A.; Frank, C.; Ernst, R. *Clin. Chem. (Winston-Salem, N.C.)* **1973**, *19*, 1361–1365.
(11) Emanuel, N. M.; Knorre, D. G. "Chemical Kinetics"; Wiley: New York, 1969; pp 174–179.
(12) Day, E. D. "Advanced Immunochemistry"; Williams and Wilkins: Baltimore, MD, 1972; pp 183–217.
(13) Hertl, W.; Odstrchel, G. *Mol. Immunol.* **1979**, *16*, 173–178.
(14) Berson, S. A.; Yalow, R S. *J. Clin. Invest.* **1959**, *38*, 1996–2016.
(15) Denizot, F. C.; Delaage, M. A. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 4840–4843.
(16) Keane, P. M.; Walker, W. H. C.; Gauldie, J.; Abraham, G. E. *Clin. Chem. (Winston-Salem, N.C.)* **1976**, *22*, 70–73.
(17) Horvath, C.; Lin, H. *J. Chromatogr.* **1978**, *149*, 43–70.

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