George, W. J., Polson, J. B., O'Toole, A. G., and Goldberg, N. D. (1970), *Proc. Natl. Acad. Sci. U.S.A. 66,* 398.
Hadden, J. W., Hadden, E. M., Haddox, M. K., and Goldberg, N. D. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 3024.
Hoffman, J. (1960), *Org. Syn. 40,* 76.
Hutton, J. and Waters, W. A. (1965), *J. Chem. Soc.,* 4254.
Illiano, G., Tell, G. P. E., Siegel, M. I., and Cuatrecasas (1973), *Proc. Natl. Acad Sci. U.S.A. 70,* 2443.
Jardetzky, C. D. (1962), *J. Amer. Chem. Soc. 84,* 62.
Kaliner, M., Orange, R. P., and Austen, K. F. (1972a), *J. Exp. Med. 136,* 556.
Kaliner, M. A., Orange, R. P., Laraia, P. J., and Austen, K. F. (1972b), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 31,* 748.
Kawazoe, Y., Maeda, M., and Nushi, K. (1972), *Chem. Pharm. Bull. 20,* 1341.
Kram, R. and Tomkins, G. M. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 1659.
Kuo, J. F. and Greengard, P. (1969), *Proc. Natl. Acad. Sci. U.S.A. 64,* 1349.
Kuo, J. F. Lee, T.-P., Reyes, P. L., Walton, K. G., Donnelly, T. E., Jr., and Greengard, P. (1972), *J. Biol. Chem. 347* 16.
Lee, T.-P., Kuo, J. F., and Greengard, P. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 3287.
Meyer, R. B., Jr., and Miller, J. P. (1974), *Life Sci. 14,* 1019.
Meyer, R. B., Shuman, D. A., Robins, R. K., Bauer, R. J., Dimmitt, M. K., and Simon, L. N. (1972), *Biochemistry 11,* 2704.
Miller, J. P., Boswell, K. H., Muneyama, K., Simon, L. N., Robins, R. K., and Shuman, D. A. (1973), *Biochemistry 12,* 5310.
Minisci, F., Bernardi, R., Bertini, F., Galli, R., and Perchinumno, M. (1971), *Tetrahedron 27,* 3575.
Minisci, F., Galli, R., Malatesta, V., and Caronna, T. (1970), *Tetrahedron 26,* 4083.
Minisci, F., and Porta, O. (1974). *Adv. Heterocycl. Chem. 16,* 123.
Muneyama, K., Bauer, R. J., Shuman, D. A., Robins, R. K., and Simon, L. N. (1971), *Biochemistry 10,* 2390.
Paoletti, R.,Berti, F., Spano, P. F., Michal, G., Weimann, G., and Nelboeck, M. (1973), *Pharmacol. Res. Commun. 5,* 87.
Puglisi, L., Berti, F., and Paoletti, R. (1971), *Experientia 27,* 1187.
Robins, R. K. (1967), *Heterocycl. Compd. 8,* 162.
Russell, T. R., Terasaki, W. L., and Appleman, M. M. (1973), *J. Biol. Chem. 248,* 1334.
Seifert, W. E., and Rudland, P. S. (1974), *Nature (London) 248,* 138.
Stoner, J., Manganiello, V. C., and Vaughan, M. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 3830.

Kinetics of Interactions between Antibodies and Haptens†

Thomas W. Smith* and Keith M. Skubitz

ABSTRACT: Association and dissociation kinetics of antibody–hapten interactions of high affinity and specificity have been determined by newly developed techniques using dextran-coated charcoal for rapid separation of free and antibody-bound hapten. Interactions of 12 combinations of four antibody populations (rabbit digoxin-specific antibody, sheep digoxin-specific antibody, rabbit ouabain-specific antibody, and rabbit digitoxin-specific antibody) with three haptens ([^{3}H]digoxin, [^{3}H]ouabain, and [^{3}H]digitoxin) have been studied in terms of both association and dissociation kinetics, and compared in selected instances with association constants determined under equilibrium conditions. Association rate constants determined under both second-order and pseudo-first-order conditions were found to be similar for all antibody–hapten pairs studied (range 0.87–1.7 $\times 10^7$ M^{-1} sec^{-1}), and were comparable to values previously estimated for antibodies to dye haptens of markedly lower affinity. In contrast, dissociation rate constants varied markedly from 1.9 $\times 10^{-4}$ to 1.7 $\times 10^{-2}$ sec^{-1}. Ratios of association to dissociation rate constants measured by these methods were in satisfactory agreement with average intrinsic association constants measured under equilibrium conditions. These studies support the concept that the major kinetic variable governing antibody–hapten interactions is the rate of dissociation of the complex, and that the strength of antibody–hapten association is determined principally by the activation energy for dissociation.

Despite the existence of an extensive literature on antibody–hapten interactions (Sehon et al., 1971) and a plethora of recent studies using such interactions as the basis for radioimmunoassay of minute concentrations of biologically active substances (Yalow, 1973; Smith and Haber, 1973), there are relatively few studies bearing on the kinetic determinants of antibody–hapten interactions. This is due in large part to the extremely rapid nature of antibody–hapten association reactions, which has necessitated the use of cathode-ray polarography (Schneider and Sehon, 1962), temperature jump relaxation (Froese et al., 1962), and spectrophotometric or fluorimetric measurements together with stopped-flow instrumentation (Day et al., 1963). Studies by Sehon and his colleagues (1963) and by Day et al.

† From the Cardiovascular Division, Peter Bent Brigham Hospital, the Cardiac Unit, Massachusetts General Hospital, and the Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115. *Received September 16, 1974.* Supported by U.S. Public Health Service Grant HL-18003. Dr. Smith is an Established Investigator of the American Heart Association.

(1963) documented rapid association kinetics with rate constants of the order of 10^7–10^8 M^{-1} sec^{-1} for antibody interactions with a phenylarsonic acid derivative and with dinitrophenyl haptens. Although direct measurements of dissociation rate constants were not made, the similarity of association kinetics in antibody–hapten interactions with differing equilibrium constants led to the hypothesis that the major kinetic variable was the dissociation rate constant, and that the strength of antibody–hapten association is determined primarily by the activation energy for dissociation (Sehon, 1971).

Our own studies have defined association constants under equilibrium conditions for high affinity interactions between cardiac glycoside-specific antibodies and the cardiac glycosides digoxin, digitoxin, and ouabain (Smith et al., 1970; Smith, 1972). In the work presented here, we have developed methods that allow the direct measurement of both association and dissociation kinetics of antibody–hapten interactions without the use of temperature-jump relaxation or stopped-flow instrumentation. The high affinity of the antibody populations for the haptens under study and the availability of tritium-labeled haptens with specific activities in the 9–20 Ci/mmol range permit dilution of reacting species to concentrations such that formation of the antibody–hapten complex can readily be followed using a simple and rapid method for the separation of free hapten from antibody–hapten complex with dextran-coated charcoal. Both association and dissociation kinetics of 12 antibody–hapten combinations have been defined, and correlated in selected instances with average intrinsic association constants determined under equilibrium conditions.

Experimental Procedures

Randomly labeled [^{3}H]digoxin (specific activity 9 Ci/mmol), [^{3}H]digitoxin (specific activity 20 Ci/mmol), and [^{3}H]ouabain (11.7 Ci/mmol) were obtained from New England Nuclear Corporation (Boston, Mass.). Purity of [^{3}H]digoxin was checked by thin-layer chromatography on silica gel G, using acetone–glacial acetic acid–cyclohexane (49:2:49); 91% of counts ran as native digoxin while 3% ran as a minor peak slightly ahead of the solvent front and the remaining counts diffusely trailed the main peak. [^{3}H]Ouabain was also tested on silica gel G, using chloroform–methanol–water (65:30:5); 96% of counts ran as a single peak with the mobility of native ouabain, the remainder trailing diffusely. Preparative thin-layer chromatography of [^{3}H]digoxin (solvent system as described previously) yielded material which gave results identical with those obtained using material as received from the supplier, and other labeled compounds were used without further purification. Tritiated compounds were diluted with absolute ethanol to appropriate concentrations before use.

Activated charcoal coated with dextran (mol wt 80,000) was prepared as described by Herbert et al. (1965), except that the ratio of charcoal and dextran to buffer was increased 2.5 times. Nonspecific rabbit γ-globulin, fraction II, was obtained from Pentex, Inc. (Kankakee, Ill.). All other reagents were of the highest available grade and were used without further purification.

Digoxin-specific antibodies were obtained as previously described in detail (Smith et al., 1970) by immunization of rabbits and sheep with digoxin–human serum albumin conjugates.

Ouabain-specific antibodies were obtained by immunization of rabbits with a conjugate of ouabain linked through its rhamnose moiety to terminal α-amino groups of poly-D,L-alanyl–human serum albumin (Smith, 1972).

Digitoxin-specific antibodies were raised in rabbits immunized with a digitoxin–human serum albumin conjugate which was synthesized according to the protocol previously published (Smith et al., 1970), substituting digitoxin for digoxin where appropriate.[1]

Antisera were separated from formed elements by centrifugation and aliquots were stored at −20°. All antisera were studied without further purification.

Studies of Association Kinetics. In order to determine the association kinetics of the various antibody–hapten combinations studied, a suitable quantity of tritiated hapten was mixed with 40 ml of 5 mg/ml of nonspecific rabbit γ-globulin in phosphate-buffered saline (0.15 M NaCl–0.01 M sodium phosphate buffer (pH 7.4)) in a 100-ml beaker. All procedures were carried out at 22.0 ±1.0°. At zero time, a small volume (0.1–1.0 ml) of antiserum appropriately diluted with phosphate-buffered saline was rapidly added to the reaction mixture, which was being stirred vigorously by a magnetic stirring bar. Parallel experiments were performed with each association kinetics determination in which all conditions were as described except that an equal volume of buffer was added in place of antibody. At time intervals, as shown in individual experiments, ranging from 5 sec to several minutes, 1.0-ml aliquots were removed with a spring-loaded syringe for separation of antibody-bound and free hapten. These aliquots were rapidly mixed with 0.5 ml of dextran-coated charcoal suspension contained in a 6-ml disposable plastic syringe, the outlet from which was plugged with a small amount of clay ("Seal-Ease," Clay Adams, Parsippany, N.J.). After 5 sec the syringe plunger was used to force the suspension rapidly through a 1.2-μ Millipore filter (Millipore Corp., Bedford, Mass.) contained in a 25-mm diameter Swinnex adapter.

After the reaction had proceeded to equilibrium, 2.5 ml of the antibody–hapten reaction mixture was mixed with an amount of undiluted antiserum specific for the hapten used such that a 10^3–10^4 ratio of antibody concentration to total hapten concentration was achieved. This mixture was allowed to react for 15 min, after which duplicate 1.0-ml aliquots were removed, mixed with charcoal suspension, and filtered as previously described. Hapten present in the filtrate under these conditions of vast excess of high affinity antibody was taken as total hapten concentration initially present (H_0).

In the case of antibody–hapten interactions with rapid dissociation kinetics, the charcoal contact time was reduced to 2 sec. Separate experiments showed charcoal binding of free hapten to be more than 97% complete within 2 sec of charcoal contact time. Since the dissociation kinetics were relatively slow for the antibody–hapten interactions under investigation (see below), the 2–5-sec charcoal exposure times did not appreciably disturb the system during separation of bound and free hapten.

One milliliter of each filtrate was added to 15 ml of liquid scintillation medium (Instagel, Packard Instrument Corp., Downers Grove, Ill.) and counted in a liquid scintillation spectrometer (Packard Model 3320).

In order to correct filtrate count rates for counter background (b), and for small contributions from free hapten and nonhapten counts not bound by charcoal, additional ex-

[1] Antiserum kindly provided by Dr. William Stason.

periments were performed. Varying amounts of hapten were mixed with 5 mg/ml of nonspecific rabbit γ-globulin in phosphate-buffered saline under conditions identical with those used in each association kinetics experiment; 1-ml aliquots were subjected to dextran-coated charcoal exposure and Millipore filtration as previously described. In the absence of antibody, count rates in the filtrate after charcoal exposure were linearly related to total hapten concentration present (Figure 1). Because of the fact that free hapten concentrations vary continuously from the start of the binding reaction until equilibrium is reached, the relation defined in plots of the type shown in Figure 1 was used to calculate the amount of hapten escaping charcoal binding at any point during the association reaction. The contribution to total filtrate count rate at time t from actual antibody–hapten complex, AbH, was calculated from the expression

$$\mathrm{AbH} = (\mathrm{AbH})_c - b - \frac{m}{1-m}[(\mathrm{T})_c - (\mathrm{AbH})_c]$$

where $(AbH)_c$ is the uncorrected filtrate count rate at time t, b is a background value for the particular counting system used, $(T)_c$ is the uncorrected filtrate count rate obtained in the presence of 10^3–10^4-fold antibody excess, m is the slope of the plot of $(B)_c$ vs. $(T)_c$, and $(B)_c$ is the total filtrate count rate observed in the absence of specific antibody. The derivation of this expression is discussed in detail elsewhere (Skubitz and Smith, 1975). In practice, the total correction applied did not exceed 10% of raw filtrate count rates.

The reaction between antibody and hapten may be written

$$\mathrm{Ab} + \mathrm{H} \underset{k_r}{\overset{k_f}{\rightleftharpoons}} \mathrm{AbH}$$

where $d(AbH)/dt = k_f(Ab)(H) - k_r(AbH)$ and $K_{ass} = k_f/k_r$. (Ab), (H), and (AbH) represent the concentrations of antibody, free hapten, and antibody–hapten complex, respectively, at time t. Antibody and hapten concentrations at the inception of the binding reactions are designated Ab_0 and H_0.

If the dissociation reaction is very slow compared with the association reaction, $d(AbH)/dt = k_f(Ab)(H) = k_f(Ab_0 - AbH)(H_0 - AbH)$. Integrating $k_f t = [1/(Ab_0 - H_0)] \ln [H_0(Ab_0 - AbH)/Ab_0(H_0 - AbH)]$ or $k_f(Ab_0 - H_0)t = \ln (H_0/Ab_0) + \ln [(Ab_0 - AbH)]/(H_0 - AbH)]$.

Two sets of reaction conditions were used to determine k_f. In the first approach, a ratio of H_0/Ab_0 of approximately 2 was used. Ab_0 was determined by measurement of filtrate count rates in the presence of hapten excess, as in the case of equilibrium studies as described subsequently. Using this method a plot of $\ln [(Ab_0 - AbH)/(H_0 - AbH)]$ vs. t should, for a single class of second-order association events, yield a straight line with slope $k_f(Ab_0 - H_0)$ and intercept $\ln (H_0/Ab_0)$.

In the second method of determining k_f, a substantial excess of hapten over antibody was used, with ratios of H_0/Ab_0 ranging from 5 to 30. Under these conditions of hapten excess, the value $(H_0 - AbH)$ is approximately constant throughout the reaction, and one may rewrite $d(AbH)/dt = k_f(Ab_0 - AbH)(H_0 - AbH)$ as a pseudo-first-order reaction, i.e., $d(AbH)/dt = k_f H_0(Ab_0 - AbH)$. Integrating, $(Ab_0 - AbH) = Ab_0 e^{-k_f H_0 t}$ or $\ln (Ab_0 - AbH) = \ln Ab_0 - k_f H_0 t$. Thus, a plot of $\ln (Ab_0 - AbH)$ vs. t should, for a single class of association events, yield a straight line with the slope equal to $-k_f H_0$. H_0 was determined experimentally in the presence of antibody excess as described above.

Studies of Dissociation Kinetics. Two methods were developed to determine the dissociation kinetics of the antibody–hapten interactions under study. All procedures were at 22.0 ± 1.0°. In the first approach, antibody–hapten complex was incubated in the presence of dextran-coated charcoal, which served to sequester free hapten as it dissociated from the complex.

Suitable amounts of antiserum were allowed to interact with tritiated hapten sufficient to provide a two- to threefold excess of hapten over specific antibody binding sites in a volume of 20 ml of 30 mg/ml nonspecific rabbit γ globulin in phosphate-buffered saline. Concentrations of antibody–hapten complex before beginning the dissociation reaction were in the 1–5 nM range. At time zero, the above mixture was added to 20 ml of the dextran-coated charcoal suspension previously described, continuously stirring with a magnetic stirring bar. Immediately after mixing and at intervals varying from 10 sec to an hour, depending on the rapidity of the dissociation reaction, 1.5-ml samples were withdrawn, the charcoal was separated by Millipore filtration, and 1.0-ml aliquots of filtrate were counted as described above. Parallel experiments were carried out at the time of each study in which all conditions were identical except that an equal volume of normal rabbit serum was substituted for antiserum, permitting the determination of blank values which were subtracted from count rates observed at each point on the dissociation curve. Human plasma obtained from the blood bank and diluted with an equal volume of phosphate-buffered saline proved to give identical results when used in place of 30 mg/ml of nonspecific rabbit γ-globulin solution, and was used as the reaction vehicle in the majority of studies.

The second approach involved the addition of a 10^3–10^4-fold excess of unlabeled hapten to an equilibrated solution of antibody–tritiated hapten complex, followed by separation of free hapten from antibody–hapten complex in aliquots taken from the solution at intervals. Due to the very limited solubility of digitoxin in aqueous solution, this method was used only for ouabain and digoxin dissociation studies. Antibody–hapten complex was formed exactly as in the previously described method. At time zero, a 10^3–10^4-fold excess of unlabeled hapten was added to the stirring mixture. Immediately after mixing and at intervals as noted above, 1.0-ml aliquots were removed and mixed for 5 sec with 0.5 ml of dextran-coated charcoal. After separation of charcoal by Millipore filtration, 1.0-ml aliquots were subjected to liquid scintillation counting. Parallel studies were again performed under identical conditions, save for the substitution of normal rabbit serum for antiserum, and values obtained were subtracted from corresponding count rates for points on the dissociation curve.

Equilibrium Studies. For certain antibody–hapten combinations, equilibrium binding studies were also performed and subjected to Sips (Karush, 1956; Nisonoff and Pressman, 1958; Eisen, 1964) and Scatchard (1949) analyses. Concentrations of antibody-bound and free hapten were determined as previously described in the case of association kinetics experiments following incubation for 1 to 2 hr at 22.0 ± 1.0° of suitable constant amounts of antiserum with varying concentrations of tritiated hapten. Initial antibody concentration was determined from AbH complex concentration in the presence of hapten excess as previously described (Smith, 1972). In Scatchard analyses the relation-

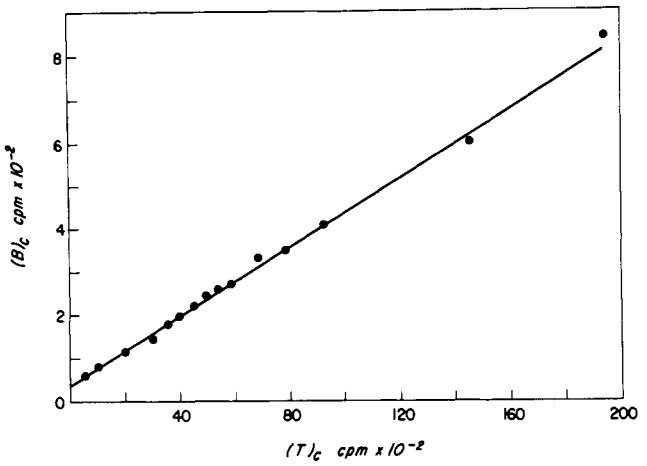

FIGURE 1: Relation of filtrate count rate after exposure to dextran-coated charcoal in the absence of antibody, designated $(B)_c$, to total hapten count rate in the filtrate in the presence of 10^4-fold antibody excess, $(T)_c$, for [^{3}H]digoxin experiments. Values for the slope of this function, designated m, varied by as much as a factor of 2 depending on the particular lot of charcoal and hapten used.

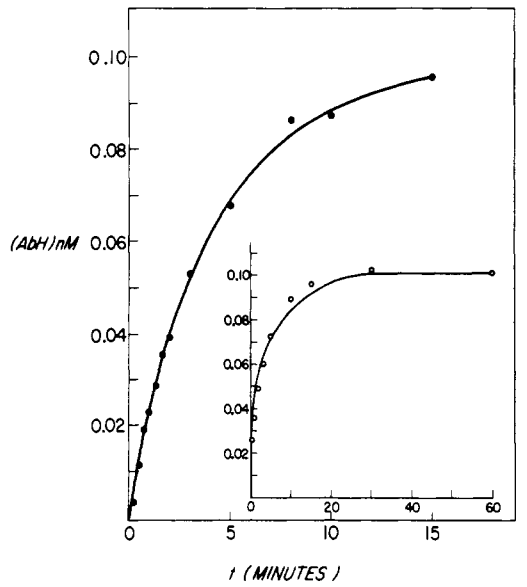

FIGURE 2: Kinetics of formation of sheep digoxin-specific antibody-[^{3}H]digoxin complex (AbH). The insert shows the plateau of filtrate count rate as equilibrium is reached. $H_0 = 2.49 \times 10^{-10}$ M; $Ab_0 = 1.15 \times 10^{-10}$ M.

ship $r/c = nK_{ass} - K_{ass}r$ where r = bound hapten/total Ab, n = antibody valence, and c = free hapten concentration, was plotted as r/c vs. r. In the Sips analysis, the relation ln $[r/(1-r)] = a \ln c + a \ln K_0$ was plotted as ln $[r/(1-r)]$ vs. ln c.

Statistics. Lines of best fit for all linear functions were determined by least-squares linear regression analysis with the aid of Sigma III and IBM 360-65 computers.

Results

Association Kinetics. The method used to correct filtrate count rates for "leakage" of free hapten and nonhapten counts during the charcoal separation of free from antibody-bound hapten depends on a predictable relation between filtrate counts in the absence of antibody, $(B)_c$, and total hapten counts present, $(T)_c$, as defined by filtrate

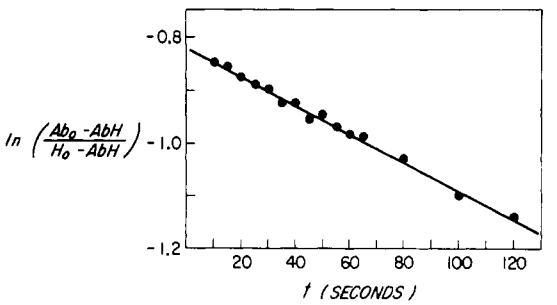

FIGURE 3: Data from Figure 2 plotted according to second-order derivation as described in text. The association rate constant from this plot is 2.0×10^7 M^{-1} sec^{-1}. The correlation coefficient for the line obtained by least-squares linear regression analysis is 0.996.

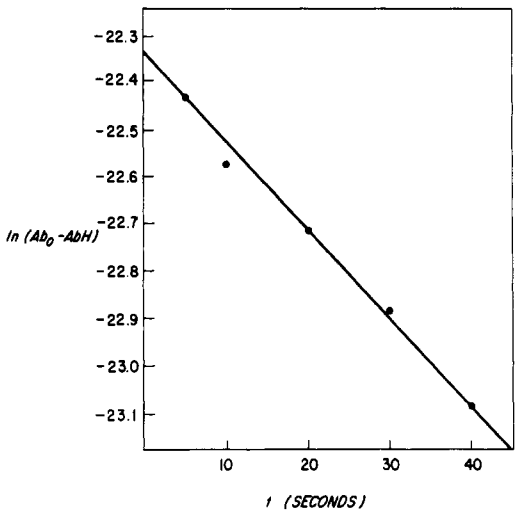

FIGURE 4: Association kinetics for sheep digoxin-specific antibody-[^{3}H]digoxin combination plotted according to pseudo-first-order derivation as described in text. The association rate constant determined from this plot was 1.6×10^7 M^{-1} sec^{-1}. The correlation coefficient for the line obtained by least-squares linear regression analysis is 0.996. $H_0 = 1.26 \times 10^{-9}$ M; $Ab_0 = 2.40 \times 10^{-10}$ M.

count rates in the presence of 10^3-10^4-fold molar excess of high affinity antibody. As shown in Figure 1, this relation was found to be linear over the range of interest and could be defined as $(B)_c = b + m(T)_c$. The values b and m were used to determine actual AbH concentration from raw filtrate count rates according to the previously stated formula.

A typical plot of AbH vs. t for the formation of complex between sheep digoxin-specific antibody and [^{3}H]digoxin is shown in Figure 2. A twofold excess of hapten was present, and second-order kinetics are apparent in Figure 3, which plots these same data as ln $[(Ab_0 - AbH)/(H_0 - AbH)]$ vs. t. Relative homogeneity of association kinetics is apparent from the linearity of this plot.

Figure 4 illustrates the results of another experiment with the same antibody-hapten pair, using pseudo-first-order analysis of data obtained in the presence of a 5.2-fold excess of hapten over antibody. As in the second-order analysis, a single class of antibodies with respect to k_f is suggested by the linearity of the plot. The value of 2.0×10^7 M^{-1} sec^{-1} obtained from the second-order treatment is in reasonable agreement with the value 1.6×10^7 M^{-1} sec^{-1} obtained from the pseudo-first-order plot.

The interaction of rabbit ouabain specific antibody with [^{3}H]ouabain was also studied under conditions of similar Ab_0 and H_0 values as well as in the presence of a 7.5-fold

Table I: Association Rate Constants for 12 Antibody–Hapten Combinations.[a]

Hapten	[^{3}H]Digoxin	[^{3}H]Ouabain	[^{3}H]Digitoxin
Rabbit digoxin-specific antibody	0.93	1.5	1.4
Sheep digoxin-specific antibody	1.7	1.6	0.98
Rabbit ouabain-specific antibody	1.3	0.87	1.4
Rabbit digitoxin-specific antibody	1.1	1.3	1.4

[a] Rate constants are expressed as the values shown $\times 10^7$ M^{-1} sec^{-1}.

Table II: Dominant Dissociation Rate Constants for 12 Antibody–Hapten combinations.[a]

Hapten	[^{3}H]Digoxin	[^{3}H]Ouabain	[^{3}H]Digitoxin
Rabbit digoxin-specific antibody	1.9×10^{-4}	1.7×10^{-2}	7.2×10^{-4}
Sheep digoxin-specific antibody	3.5×10^{-4}	1.5×10^{-2}	1.9×10^{-3}
Rabbit ouabain-specific antibody	6.4×10^{-3}	1.5×10^{-3}	3.8×10^{-3}
Rabbit digitoxin-specific antibody	1.2×10^{-3}	1.4×10^{-2}	2.3×10^{-4}

[a] Rate constants are expressed in units of sec^{-1}.

excess of hapten over antibody, and data obtained were analyzed by second-order and pseudo-first-order treatment. Values for k_f obtained were 0.79×10^7 and 0.83×10^7 M^{-1} sec^{-1} for second-order and pseudo-first-order conditions, respectively, and were in satisfactory agreement. Since the pseudo-first-order reaction was simpler to analyze, other antibody–hapten combinations were studied using this approach.

The results for association kinetics studies of all 12 combinations of the four antibody populations and three haptens are summarized in Table I. The values given represent the average values of at least two separate experiments for each antibody–hapten combination studied except for the rabbit digitoxin-specific antibody–[^{3}H]digoxin combination, where the value listed is from a single determination. Agreement was within 28% in all but two instances, the range for which was 46%. The mean range for replicate samples was 19%.

It is apparent from these data that association rate constants for all 12 antibody–hapten combinations are quite similar, despite pronounced differences in dissociation rate constants and equilibrium constants as discussed subsequently.

Dissociation Kinetics. A comparison of the two methods for the determination of k_r values is shown in Figure 5. Dissociation of [^{3}H]digoxin from rabbit digoxin-specific antibody is plotted as percent of initial AbH complex vs. time and demonstrates good agreement between data from prolonged exposure to dextran-coated charcoal and addition of a large excess of unlabeled digoxin. Equally good agreement of data obtained by these two approaches was observed for dissociation of [^{3}H]digoxin from sheep digoxin-specific antibodies and for dissociation of [^{3}H]ouabain from rabbit ouabain-specific antibodies. Figure 5 also demonstrates marked differences in the rates of dissociation of

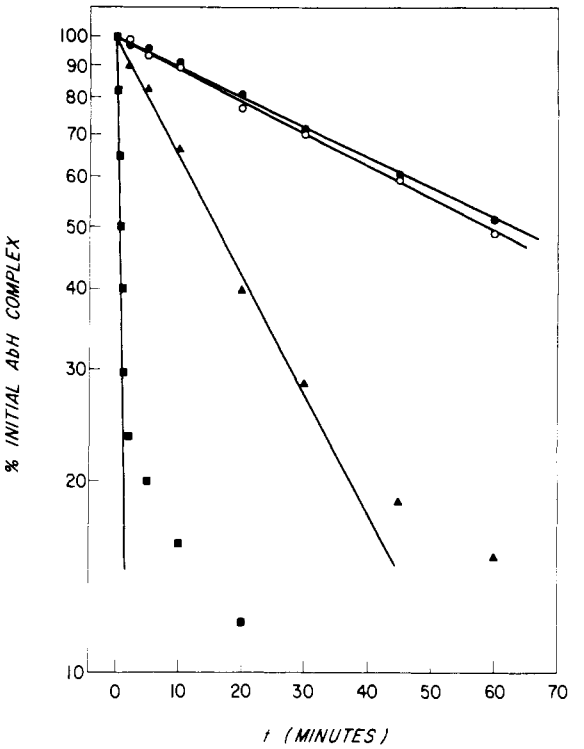

FIGURE 5: Dissociation kinetics for complex of rabbit digoxin-specific antibody with [^{3}H]digoxin (circles), [^{3}H]digitoxin (triangles), and [^{3}H]ouabain (squares). Dissociation kinetic data for [^{3}H]digoxin are shown both for the prolonged charcoal exposure method (closed circles) and following addition of a 10^3-fold excess of unlabeled digoxin (open circles). Initial AbH complex concentration was 1.5×10^{-9} M for the [^{3}H]digoxin complex, 1.2×10^{-9} M for the [^{3}H]digitoxin complex, and 4.0×10^{-9} M for the [^{3}H]ouabain complex.

[^{3}H]ouabain and [^{3}H]digitoxin from rabbit digoxin-specific antibody compared with the dissociation rate of [^{3}H]digoxin.

Values of k_r for all 12 antibody–hapten combinations studied are summarized in Table II. Averages of two to seven individual experiments which agreed within 20% (mean variance 12%) are shown. It is evident in Table II that, in contrast to the marked similarity of forward reaction rate constants for the various antibody–hapten combinations studied, the reverse reaction rate constants varied by nearly two orders of magnitude from 1.9×10^{-4} to 1.7×10^{-2} sec^{-1}. Both unlabeled hapten excess and charcoal dissociation experiments often demonstrated slowing of the later portion of the reaction, as shown in Figure 5, consistent with some degree of heterogeneity of k_r values for the antibody populations under study. The values listed in Table II are those of the dominant class, which always comprised 65% or more of the total antibody population.

Equilibrium Studies. The rapid separation of free from antibody-bound hapten with dextran-coated charcoal also proved useful in experiments designed to determine average intrinsic association constant (K_0) values for antibody–hapten interactions. Figure 6 illustrates the Sips analysis of data for the interaction of sheep digoxin-specific antibody with [^{3}H]digoxin. The K_0 value of 2.3×10^{10} M^{-1} was identical with that obtained from a Scatchard plot, which showed a single class of binding site affinities consistent with the a value of 0.97 from the Sips plot. This K_0 value was in reasonable agreement with a value of 1.9×10^{10} M^{-1} obtained by equilibrium dialysis (data not shown) using methods previously described (Smith et al., 1970).

Table III: Previous Studies of Antibody—Hapten Interaction Kinetics.

Antibody	Hapten	k_f (M^{-1} sec^{-1})	k_r (sec^{-1})	K_0 (M^{-1})	Method	Ref
Anti-phenylarsonate	1-Naphthol-4-4-(4′-azobenzeneazo)-phenylarsonic acid	2×10^7	50		Temperature jump—spectrophotometric	Froese et al., 1962
Anti-2,4-dinitrophenyl	Dnp-lysine	8×10^7		1.3×10^8	Stopped-flow—fluorometric	Day et al., 1963
	Dnp-aminocaproate	10×10^7		1.1×10^8		
	DnpNS[a]	8×10^7		1.7×10^8		
Anti-fluorescein	Fluorescein	6.7×10^7		6.1×10^{10}	Stopped-flow—fluorometric	Levison et al., 1971
Anti-*p*-nitrophenyl	4,5-Dihydroxy-3-(*p*-nitrophenylazo)-2,7-naphthalene-disulfonic acid	1.8×10^8	7.6×10^2		Temperature jump—spectrophotometric	Froese and Sehon, 1965
Anti-2,4-dinitrophenyl	1-Hydroxy-4-(2,4-dinitrophenylazo)-2,5-disulfonic acid	1.6×10^7	80	1.5×10^5	Temperature jump—spectrophotometric	Froese, 1968
	1-Hydroxy-4-(4-phenylazo)-2,5-disulfonic acid	1.4×10^7	410	$\sim 1 \times 10^4$		
Anti-2,4-dinitrophenyl	1-Hydroxy-4-(2,4-dinitrophenylazo)-2,5-naphthalene-disulfonate	0.95×10^7	76	1.5×10^5	Temperature jump—spectrophotometric	Kelly et al., 1971
	Dnp-lysine	1.1×10^7		2.2×10^7	Stopped-flow—fluorometric	
MOPC 315	Dnp-lysine	1.3×10^8	53	2×10^6	Temperature jump—fluorometric	Pecht et al., 1972
	Dnp-glycine	1.9×10^8	1.3×10^3			

[a] DnpNS, 1-hydroxy-2-dinitrophenylazonaphthalene-3,6-disulfonic acid.

The validity of the approach described here for determination of k_r and k_f values is supported by a K_{ass} value of 4.8×10^{10} M^{-1} calculated from the relation $K_{ass} = k_f/k_r$ using directly measured k_f and k_r values for the sheep digoxin-specific antibody-[^{3}H]digoxin combination, in reasonable agreement with the values obtained under equilibrium conditions. A K_0 value of 3.5×10^9 M^{-1} from Sips analysis of equilibrium data for the rabbit ouabain-specific antibody-[^{3}H]ouabain interaction was also in satisfactory agreement with a k_f/k_r ratio of 5.8×10^9 M^{-1} sec^{-1}.

Discussion

Data from studies using temperature-jump and stopped-flow techniques indicate that antibody–hapten association reactions are too rapid to be delineated by conventional chemical kinetic methods unless the concentrations of reacting species can be reduced to levels of the order of 10^{-8}–10^{-10} M (Froese and Sehon, 1971). In the present study we have taken advantage of the availability of tritium-labeled haptens of high specific activity and radiochemical purity, antibody populations of high affinity for the haptens under study, and a newly developed method for the rapid separation of antibody-bound from free hapten utilizing dextran-coated charcoal. Our technique has allowed us to study the kinetics of antibody–hapten interactions at concentrations of both antibody and hapten of the order of 10^{-9}–10^{-10} M, placing these interactions well outside the range where the rate of mixing of reactants is the rate-limiting step (Wang, 1955). This experimental approach obviates the need for complex and expensive temperature-jump and stopped-flow apparatus and permits the study of haptens which do not possess special optical properties, unlike haptens previously subjected to kinetic investigation (Table III). An additional advantage of this method is the lack of need to isolate the antibody population, or at least the γ globulin fraction, prior to study. The use of the entire antibody population present in crude antiserum in kinetic studies permits more straightforward assessment of heterogeneity of the immune response of a given animal than if prior fractionation techniques have been employed. It should be noted, however, that in using antibody and hapten concentrations less than 10^{-9} M the contribution to the measured reaction rate by antibodies with affinity constants less than 10^8 M^{-1} would be negligible.

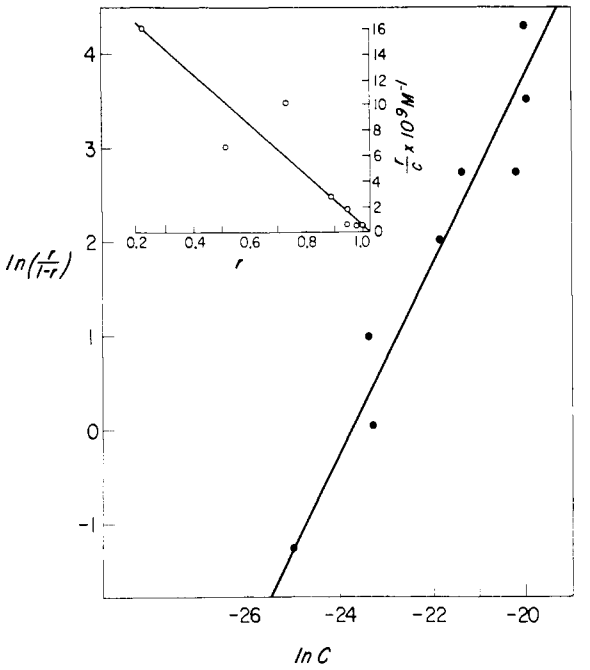

FIGURE 6: Sips analysis of equilibrium binding data for sheep digoxin-specific antibody and [^{3}H]digoxin, using dextran-coated charcoal separation of bound and free hapten. K_0 is 2.3×10^{10} M^{-1}, a is 0.97, and the correlation coefficient for the line obtained by least-squares linear regression analysis is 0.960. The insert shows a Scatchard plot of the same data, which also yields an association constant of 2.3×10^{10} M^{-1}.

Data summarized in Table I clearly support previous observations (Sehon, 1963) that association kinetics for various antibody–hapten combinations differ relatively little. Table III briefly summarizes the existing literature concerning antibody–hapten interaction kinetics. The absolute values of 0.9–1.7 $\times$ 10^7 M^{-1} sec^{-1} for antibody–hapten combinations reported here are similar to values obtained by Froese et al. (1962), Froese and Sehon (1965), Froese (1968), and Kelly et al. (1971) for the antibody–hapten combinations summarized in Table III, but are roughly tenfold lower than the values obtained by Day et al. (1963), Levison et al. (1971), and Pecht et al. (1972) for the reactions listed. The relative similarity of the forward rate constants determined in our studies to those previously observed is of particular interest in view of the fact that K_0 values are one to five orders of magnitude greater than those of most antibody–hapten combinations previously subjected to kinetic study.

In contrast to the relative similarity of k_f values for the 12 antibody–hapten combinations studied, Table II documents an 89-fold variation in dissociation rate constants. These data further support the previous findings of Froese (1968) and Pecht et al. (1972). Since the specificities of antibody–hapten interactions are fundamentally determined by equilibrium constants of the interactions involved, it follows that the dissociation rate constant is the principal variable governing antibody–hapten specificity.

The forward and reverse rate constants we report here, in contrast to those reported earlier for other antibody–hapten systems (Table III), were independently determined in separate experiments. The validity of absolute k_f and k_r values determined in these studies is supported by the similarity of K_0 values determined under equilibrium conditions, e.g., Figure 6, and k_f/k_r ratios determined in kinetic experiments for both sheep digoxin-specific antibody–[^{3}H]digoxin and rabbit ouabain-specific antibody–[^{3}H]ouabain interactions.

Day et al. (1963) have suggested that the antibody–hapten association reaction may be diffusion limited. Froese and Sehon (1965) have calculated a maximum k_f value of about 1.5 $\times$ 10^9 M^{-1} sec^{-1} using relationships derived by Alberty and Hammes (1958). They point out, however, that this estimate does not take electrostatic repulsive forces into account and suggest that the true theoretical diffusion limited value of k_f may be tenfold slower, or about 10^8 M^{-1} sec^{-1}. This value still exceeds the directly determined association rate constants in the present work by about an order of magnitude.

Although Sips analysis of equilibrium data for the rabbit ouabain-specific antibody–[^{3}H]ouabain and sheep digoxin-specific antibody–[^{3}H]digoxin combinations yielded values close to unity for the heterogeneity constant, a, the dissociation experiments showed a definite heterogeneity of the antibody populations with respect to dissociation reaction rate constants. It may be that the 2-hr incubation time used in equilibrium experiments did not permit full equilibration at the lowest hapten concentrations, thus tending to underestimate true heterogeneity by this method. Evidence for a more slowly dissociating subpopulation of less than 35% of total specific antibody was observed using methods involving both prolonged charcoal exposure and addition of a large excess of unlabeled hapten. It is therefore unlikely that this apparent heterogeneity was artifactual. In contrast to the heterogeneity of association kinetics systems previously studied by Day et al. (1963), we detected no significant heterogeneity of k_f values.

Day et al. (1963) have suggested that the similarity of results observed for the interaction of both the zwiterionic hapten, Dnp-lysine, and the anionic hapten, Dnp-aminocaproate, implies that it is not the heterogeneity of net ionic charge of isolated anti-Dnp antibody which is primarily responsible for the kinetic heterogeneity of the antibody population studied. While the studies of antibody–hapten interactions reported earlier were performed with relatively polar, charged haptens, our studies show similar values of k_f for the uncharged nonpolar, hydrophobic haptens digoxin and digitoxin as well as for the relatively polar cardiac glycoside, ouabain. This indicates that the electrostatic charge and polarity of the hapten, at least to the extent of the variation in charge and polarity yet studied, have little effect on the value of forward reaction rate constants.

References

Alberty, R. A., and Hammes, G. G. (1958), *J. Phys. Chem. 62,* 154.

Day, L. A., Sturtevant, J. M., and Singer, S. J. (1963), *Ann. N.Y. Acad. Sci. 103,* 611.

Eisen, H. N. (1964), *Methods Med. Res. 10,* 106.

Froese, A. (1968), *Immunochemistry 5,* 253.

Froese, A., and Sehon, A. H. (1965), *Immunochemistry 2,* 135.

Froese, A., and Sehon, A. H. (1971), *Methods Immunol. Immunochem. 3,* 412.

Froese, A., Sehon, A. H., and Eigen, M. (1962), *Can. J. Chem. 40,* 1786.

Herbert, V., Lau, K. S., Gottleib, C. W., and Bleicher, S. J. (1965), *J. Clin. Endocrinol. Metab. 25,* 1375.

Karush, F. (1956), *J. Am. Chem. Soc. 78,* 5519.

Kelly, K. A., Sehon, A. H., and Froese, A. (1971), *Immunochemistry 8,* 613.

Levison, S. A., Portmann, A. J., Kierszenbaum, F., and Dandliker, W. B. (1971), *Biochem. Biophys. Res. Commun. 43,* 258.

Nisonoff, A., and Pressman, D. (1958), *J. Immunol. 80,* 417.

Pecht, I., Givol, D., and Sela, M. (1972), *J. Mol. Biol. 68,* 241.

Scatchard, G. (1949), *Ann. N.Y. Acad. Sci. 51,* 660.

Schneider, A. H., and Sehon, A. H. (1962), *Trans. N.Y. Acad. Sci. 24,* 15.

Sehon, A. H. (1963), *Ann. N.Y. Acad. Sci. 103,* 626.

Sehon, A. H. (1971), *Methods Immunol. Immunochem. 3,* 375.

Sehon, A. H., Karush, F., Karush, S. S., Eisen, H. N., McGuigan, J. E., Froese, A., Cebra, J. J., and Dandliker, W. B. (1971), *Methods Immunol. Immunochem. 3,* 375.

Skubitz, K. M., and Smith, T. W. (1975), *J. Immunol.* (in press).

Smith, T. W. (1972), *J. Clin. Invest. 51,* 1583.

Smith, T. W., Butler, V. P., Jr., and Haber, E. (1970), *Biochemistry 9,* 331.

Smith, T. W., and Haber, E. (1973), *Prog. Cardiol. 2,* 49.

Wang, J. H. (1955), *J. Phys. Chem. 59,* 1115.

Yalow, R. S. (1973), *Circ. Res., Suppl. 1,* 116.