KINETIC STUDIES OF A DOUBLY BOUND RED CELL ANTIGEN-ANTIBODY SYSTEM

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ABSTRACT The Polybrene method for detection of red cell antibodies which utilizes continuous flow equipment was modified so that kinetic studies could be performed on red cell antibodies doubly bound between adjacent red cells. In the anti-Rh_o-Rh_o erythrocyte system, deaggregation by temperature was studied over an antibody concentration range of from approximately 1 to 500 antibody molecules per erythrocyte, a residence time range of approximately eightfold, and a temperature range of from 10 to 55°C. The rate of dissociation of antigen-antibody complex, as determined from deaggregation of antibody-dependent red cell aggregates, was found to be of apparent zero order. The apparent activation energy for the antigen-antibody reaction under the experimental conditions was determined and found to be higher than would be expected for singly bound antigen-antibody systems. Possible explanations are considered for these findings in terms of an antigen-antibody bond-breaking model.

INTRODUCTION

In spite of wide interest in red cell antigen-antibody reactions, the available information on the physicochemical aspects of these reactions has been scant. Recently it has been reported that positively charged polymers such as Polybrene® (polyhexadimethrine bromide) may be used for detection of red cell antibodies with a high degree of sensitivity (1). In this communication, a modification of the Polybrene method was employed to study the kinetics of antigen-antibody reactions when γG (7S) antibody molecules were doubly bound between adjacent red cells. Until the advent of the Polybrene technique, such reactions were not amenable to study.

EXPERIMENTAL METHODS

The study was carried out in a continuous flow analyzer (Fig. 1). This device may be considered as a tubular chemical reactor operating at steady state in which portions of well stirred reaction mixture, separated by bubbles of air, can be maintained under controlled

conditions for controlled periods of time. In this device, gentle stirring is provided by the use of coiled glass tubing, since mechanical agitators could damage the cells. Special fittings are provided for the addition of reactants and for air to segment the liquid flow, and decanters are provided for removing components which have settled to the bottom of the tubes after mixing has been stopped. Temperature is controlled by external baths, and residence time is controlled by varying the length of the tubes. The pumping rate cannot be varied, since the optimum operation of various portions of the continuous flow system, for example the decanters, depends upon maintaining a constant pumping rate. More detailed information concerning the continuous flow system may be found elsewhere (1, 2).

In brief, the method involves several independent steps. The first step in the reaction sequence is the uptake of antibodies of known concentration by washed red cells in an acidified low ionic medium. Under low ionic conditions, the rate of uptake of the antibody molecules is greatly accelerated (3). In order to insure a uniform antibody distribution, red cells are first mixed with antibody and then introduced into the low ionic medium. Since the red cell surface is negatively charged because of ionization of carboxylic acid groups that constitute part of the red cell membrane, repulsive forces exist which prevent the cells from approaching more closely than an estimated distance of the order of 250 A (4). Most 7S antibodies are too "short" to bridge across this distance. The addition of Polybrene results in a powerful but reversible agglutination of the red cells. This allows antibody molecules which have been singly bound to the red cells to bridge between antigen sites on adjacent cells and become doubly bound. The Polybrene agglutination is apparently the result of neutralization of the negative surface charge on the red cells. Discussion of the Polybrene-red cell interaction may be found elsewhere (5, 6). This agglutination is reversed by the addition of hypertonic sodium citrate. In the absence of antibodies, the aggregates disperse and the cells resuspend. In the presence of antibodies all or part of the aggregates persist, depending upon the amount of antibody present. The aggregates are then allowed to settle and are removed by means of a decanter. The lengths of mixing and settling coils required for optimal sensitivity had previously been determined experimentally (1). The remaining unaggregated cells are hemolyzed with a detergent and passed through a colorimeter for quantitation of hemoglobin. The hemoglobin detected is thus inversely related to the amount of antibody introduced into the system.

Type O, R_1r red cells were freshly collected each week from a normal donor in citrate-phosphate-dextrose (CPD) anticoagulant (1) and stored at 4°C. On each work day, a sample was washed three times in 0.9% NaCl and suspended in a 0.9% NaCl solution which also contained 0.25% (w/v) Ficoll, a polysaccharide that reduces the amount of hemolysis in the test system (7). On the basis of a red cell count by means of the Coulter Model S cell counter (Coulter Electronics, Inc., Hialeah, Fla.), the cell suspension was diluted accordingly to obtain 8.0×10^8 red cells/ml (hematocrit of about 6.5%).

Normal plasma was separated from different Rh positive blood units that had been collected in acid-citrate-dextrose (ACD) anticoagulant for use in the blood bank, pooled, and recalcified to obtain a large supply of serum. Part of this serum was used to prepare various dilutions of an anti-Rh_o antibody[1] (anti-D) which had previously been assayed at 13 μg/ml by two independent methods, using ^{125}I-tagged antibody (8) and automated polyvinylpyrrolidone (PVP)-bromelin (9) techniques.

The system was studied over an antibody concentration range of from approximately 1 to approximately 500 antibody molecules per red cell introduced into the continuous flow

[1] This antibody was supplied through the courtesy of Dr. B. P. L. Moore, Director, National Reference Laboratories, Canadian Red Cross Blood Transfusion Service, Toronto, and had been quantitated by Doctors Moore and Hughes-Jones.

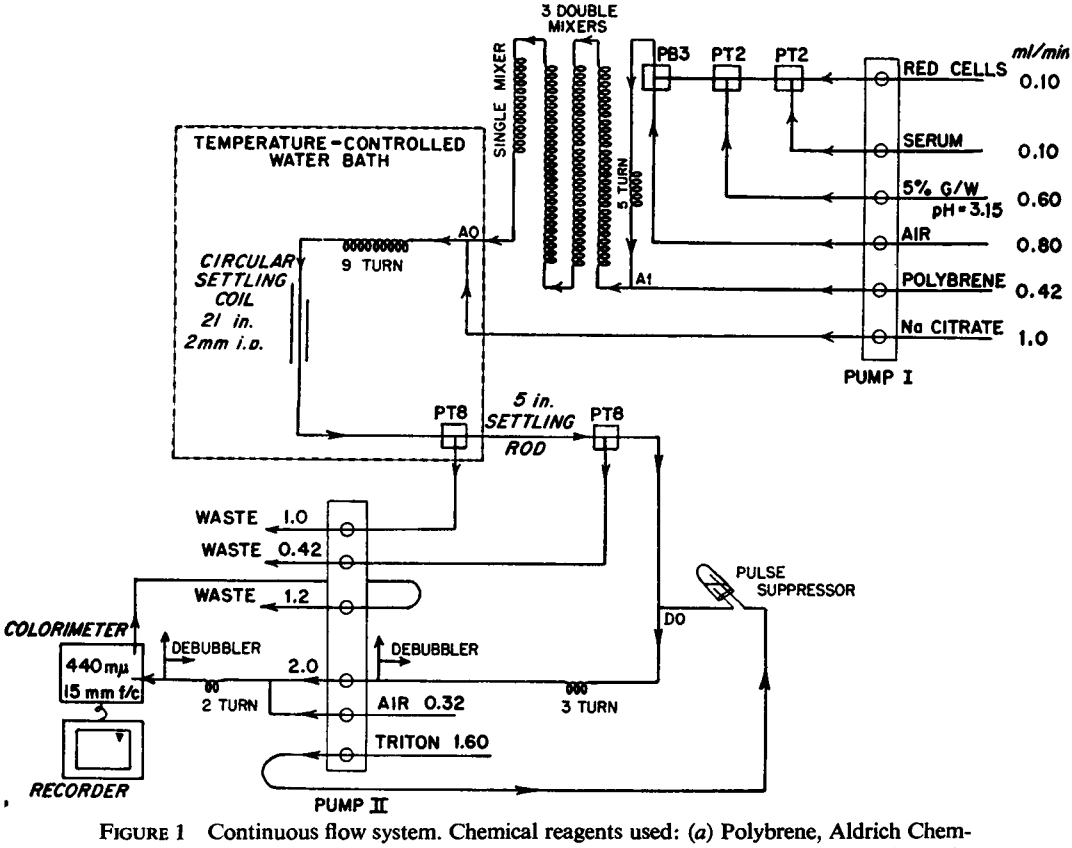


FIGURE 1 Continuous flow system. Chemical reagents used: (*a*) Polybrene, Aldrich Chemical Co., Inc., Milwaukee, Wis., lot 31551. Stock solution, 100 mg/ml in 0.9% NaCl. Working solution (pH 6.6), diluted 1:250 with 0.9% NaCl. (*b*) Acidified glucose (pH 3.15): 0.1 ml of 1 M citric acid solution added to 50 ml of 5% glucose in distilled water. (*c*) Resuspending solution: stock solution, 1 M trisodium citrate. Working solution (pH 8.2), 2 vol 1 M sodium citrate plus 8 vol distilled water. (*d*) Triton X-100, Technicon Co., Inc., Tarrytown, N. Y., 0.5% in distilled water. Component fittings PB3, PT2, PT8, and AO deliver air and reactants into the main flow stream. Components for the continuous flow analyzer were obtained from Technicon Co., Inc. For temperature control, a thermoregulated circulator Neslab Model RTE 3-Z, obtainable from Neslab Instruments, Inc., Portsmouth, N.H., was used in conjunction with a water bath. *Note:* In the kinetic studies, the resuspension coil (9-turn coil shown above) was replaced by 10-, 29-, 53-, and 84-turn coils. The flow rates were approximately 75% of the values shown in the above diagram since slower 10-roller pumps were employed. The values in the diagram are commercial values obtained by the producer when 5-roller pumps are used.

analyzer as determined by input flow rates, red cell counts, and antibody assays. While the two initial phases of antibody uptake and Polybrene-induced aggregation were maintained at room temperature (24°C), the Polybrene neutralization phase was allowed to occur at different temperatures and lengths of time. Thus, the reaction was studied over a range of approximately eightfold in residence time and a temperature range of from 10.0 to 55.0°C. The residence time was changed by changing the length of the coils involved in the deaggregation phase, and temperature was controlled by the use of a water bath (Fig. 1).

RESULTS

A typical experimental result, taken at 10.0°C with 10 turns of helical coil in the deaggregation phase, is shown in Fig. 2. Different antibody concentrations are represented. To obtain these data, the red cells were sent continuously through the system. Each antibody concentration was sampled for 5 min to achieve steady state

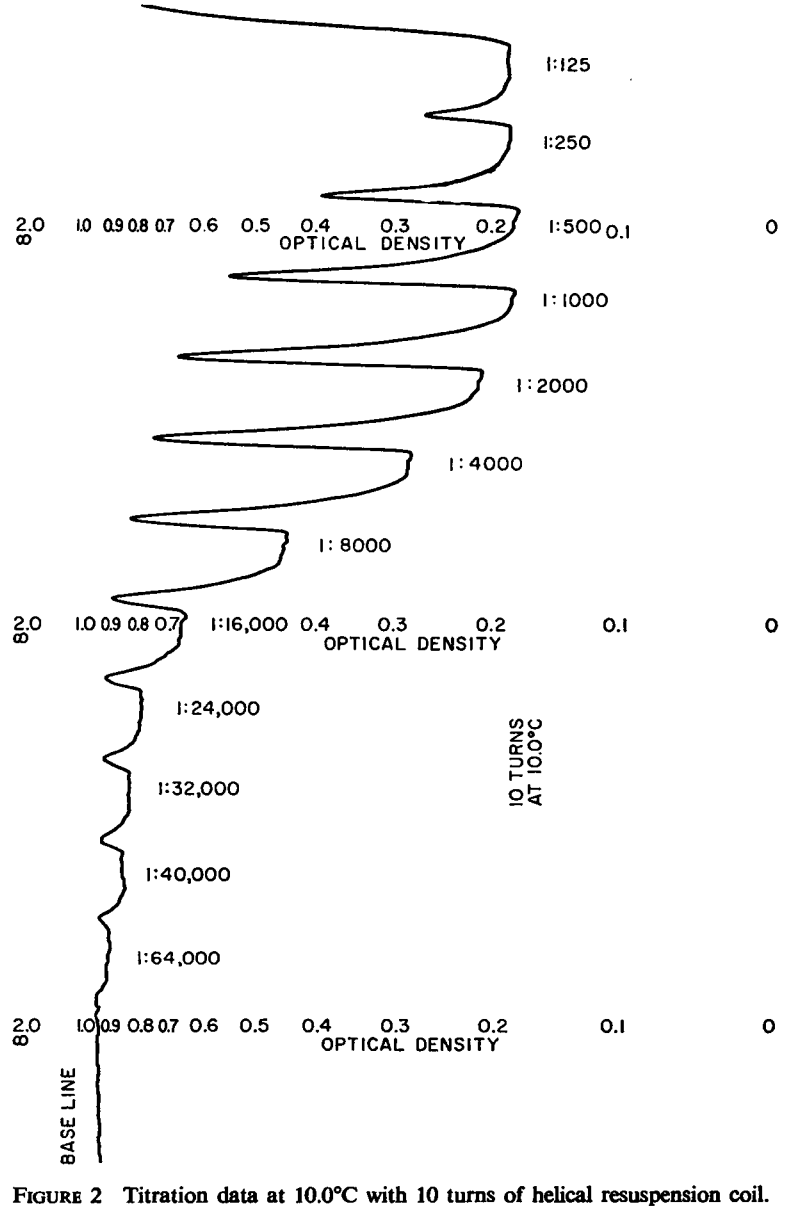


FIGURE 2 Titration data at 10.0°C with 10 turns of helical resuspension coil.

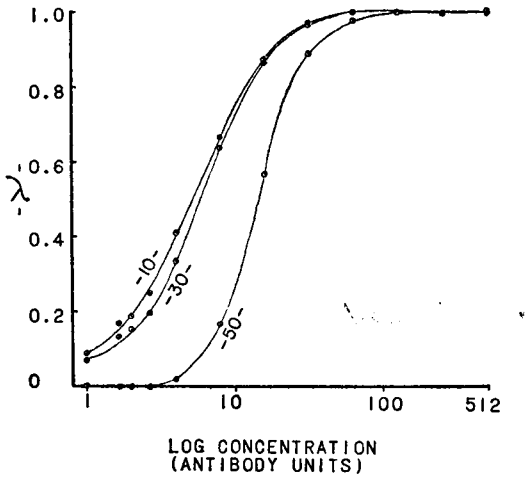


FIGURE 3 Fraction of cells aggregated (λ) vs. log concentration of the antibody in antibody units at 10.0, 30.0, and 50.0°C with 10 turns of helical resuspension coil. (A 1:64,000 dilution of antibody was selected to represent one antibody unit and corresponded to approximately one antibody molecule per red cell entering the continuous flow system.)

and followed by a 2 min wash of normal serum. The base line was obtained by sampling normal serum for a half-hour. It can be seen from the inverted plateaus of the figure that optical density decreased progressively as the number of antibody molecules added to the system was increased, until the saturation value was reached. At saturation, essentially all of the red cells were agglutinated and decanted. At the lowest antibody concentrations used, only a negligible number of the red cells were agglutinated.

Fig. 3 shows a plot of λ(ΔOD/ΔOD max) vs. antibody cntration at 10 turnsonce for 10.0, 30.0, and 50.0°C runs. Concentration has been expressed in antibody units where a 1:64,000 dilution of the original antibody concentration was defined as one antibody unit. From material balance equations and Beer's law it can be shown that λ is equal to the fraction of cells in aggregated form. The effect of temperature on deaggregation is clearly seen in this figure. The reproducibility of the data is shown in Fig. 4, where data were taken with different preparations of the same cells on different days at 30.0°C and with 10 turns of helical coil. A high degree of reproducibility may be observed. From the resulting sigmoid curves it may be calculated that approximately five antibody molecules per red cell introduced into the flow system were required for agglutination of 50% of the cells (λ = 0.5). This was determined by measurements of input flow rates, cell counts, and antibody assay values. A mean molecular weight of 160,000 was assumed. Thus, at the lowest dilution used (1:64,000) approximately one antibody molecule per red cell was detected.

The data obtained were expressed in terms of λ at residence times corresponding to 10, 29, 53, and 84 helical coils in the deaggregation phase; temperatures of 10.0, 20.0, 30.0, 40.0, 50.0, and 55.0°C; and 12 different antibody dilutions ranging from

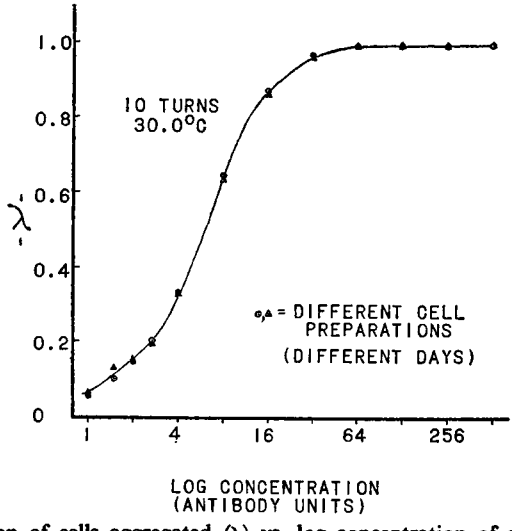


FIGURE 4 Fraction of cells aggregated (λ) vs. log concentration of antibody at 30.0°C with 10 turns of helical resuspension coil.

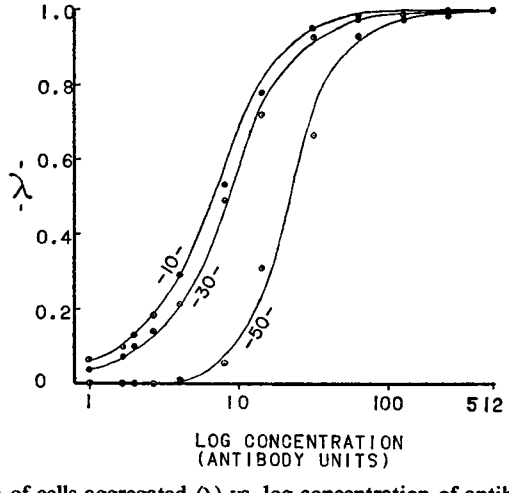


FIGURE 5 Fraction of cells aggregated (λ) vs. log concentration of antibody at 10.0, 30.0, and 50.0°C with 29 turns of helical resuspension coil.

1:125 to 1:64,000. Some of the data obtained are presented in Figs. 5–7. A comparison of Figs. 3, 5, and 6 show that the sigmoid curves shift to the right with increasing residence time (length of coil) at any temperature. This point is brought out more fully in Fig. 7, where curves at four different residence times are presented, all taken at 50°C. Alternately, one can linearize sigmoid curves such as those shown in Figs. 3–7 by plotting on log-probit paper; however, since no new information is provided by such plots and since such presentation might tend to interfere with an intuitive interpretation of the data, these plots are not given here.

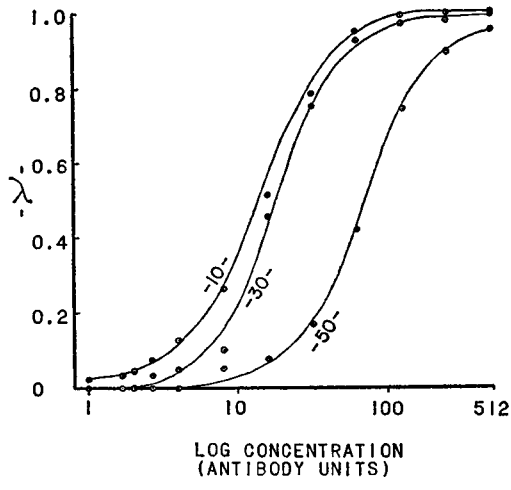


FIGURE 6 Fraction of cells aggregated (λ) vs. log concentration of antibody at 10.0, 30.0, and 50.0°C with 84 turns of helical resuspension coil.

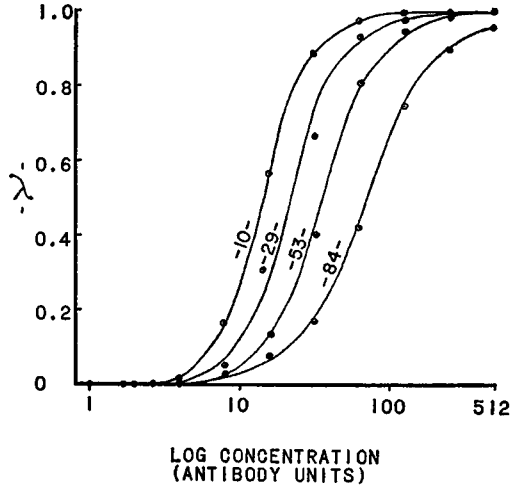


FIGURE 7 The effect of residence time (τ) on the plot of λ vs. log concentration of antibody at 50.0°C for 10, 29, 53, and 84 turns of helical resuspension coil.

INTERPRETATION OF RESULTS

Since the reaction system studied herein is not at equilibrium as evidenced by increased deaggregation with time, one cannot employ the tools usually used for interpretation of results in the widely used equilibrium dialysis methods for studying antibody-antigen interactions in solution. For this reason, new analytical tools had to be developed.

Red blood cell deaggregation at the resuspension phase appears to result from

the breaking of antigen-antibody bonds that hold the cells together. It can be assumed that all of the antibody molecules are doubly bound between adjacent cells for the following reasons:

(*a*) The number of free antibody molecules remaining after the sensitization phase is less than 1% of the number that bind to the cells under conditions of antigen excess, based upon equilibrium data for many antibodies. This condition is satisfied in the antibody concentration range used for these experiments and by the consideration that the number of antigen sites per red cell has been estimated to be of the order of 24,000 (10).

(*b*) The antibody employed cannot doubly bind to the same cell, since calculations show that the spacing between antigen sites on the cell surface is greater than the length of the antibody molecules. This assumes uniform spacing of antigen sites, and excludes the possibility that the antigen sites may be present in clusters on the red cell surface.

(*c*) The antibody may be singly bound but it is unlikely that many of the antibody molecules taken up by the cells during the sensitization phase escape double binding when the powerful Polybrene-induced hemagglutination occurs. Evidence for this last assertion is the extreme sensitivity of the test for detection of antibodies.

If we assume that for a given amount of antibody put into the system little or none is contained on the cells that have failed to aggregate, and that each of the aggregated cells contains approximately the same number of antibody molecules, then one can interpret the degree of deaggregation in terms of a specific number of bonds broken. (A test of this assumption of equal distribution of antibody molecules follows.) For example, when 50% of the initially aggregated cells have deaggregated, we will assume that half of the total number of antibody molecules have been carried along with deaggregated cells and half of the total number of bonds have been broken. Although each doubly bound antibody molecule (Ab) forms two bonds, one with each antigen site (Ag), only one of these bonds need be broken to dissociate the double complex (Ag·Ab·Ag). In this system, total number of bonds was defined as being equal to the number of such complexes and therefore equal to the number of doubly bound antibody molecules. One can thus define N_b, the apparent number of bonds broken, by the equation:

$$N_b = \left[\frac{N}{M\,\lambda i}\right][M(\lambda i - \lambda f)] = N\,[1-(\lambda f/\lambda i)], \qquad (1)$$

where N is the number of antibody molecules introduced into the system, M is the number of red cells introduced into the system, λi is the initial aggregation fraction (at $\tau = 10$), λf is the final aggregation fraction (at $\tau > 10$). The values for λi and λf are readily obtained from plots of λ vs. τ at different temperatures and antibody concentrations. Figs. 8 and 9 are examples of this type of plot for 1:500 and 1:1000 antibody dilutions. From these plots, the complex kinetic behavior of the deaggre-

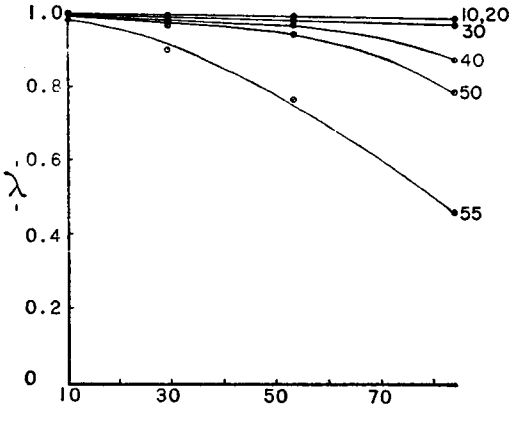


FIGURE 8

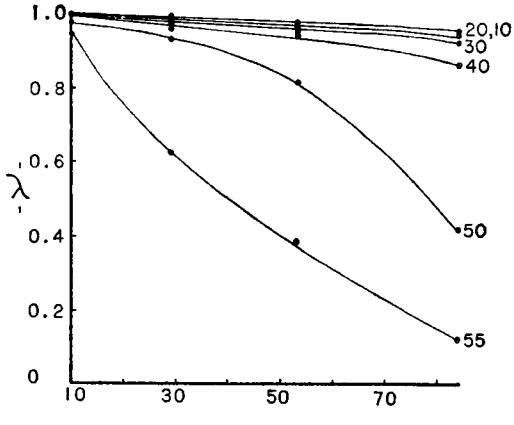


FIGURE 9

FIGURE 8 Fraction of cells aggregated (λ) vs. residence time (τ) at various temperatures for a 1:500 dilution of antibody.

FIGURE 9 Fraction of cells aggregated (λ) vs. residence time (τ) at various temperatures for a 1:1000 dilution of antibody.

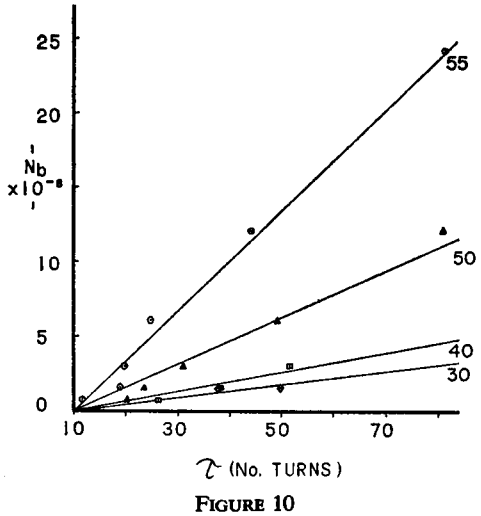


FIGURE 10

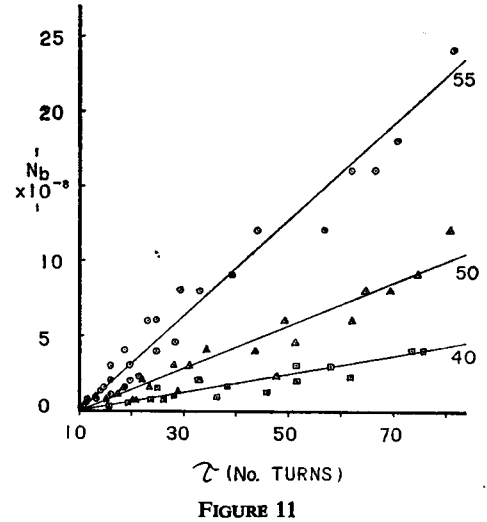


FIGURE 11

FIGURE 10 Number of bonds broken (N_b) vs. residence time (τ) at various temperatures based upon a 50% bond-breaking model.

FIGURE 11 Number of bonds broken (N_b) vs. residence time (τ) at 55.0, 50.0, and 40.0°C for composite bond-breaking model.

gation process may be visualized. Further discussion of this behavior is provided elsewhere (10).

Fig. 10 presents N_b vs. residence time (τ) expressed in number of turns at various temperatures where each point represents a different antibody concentration. The

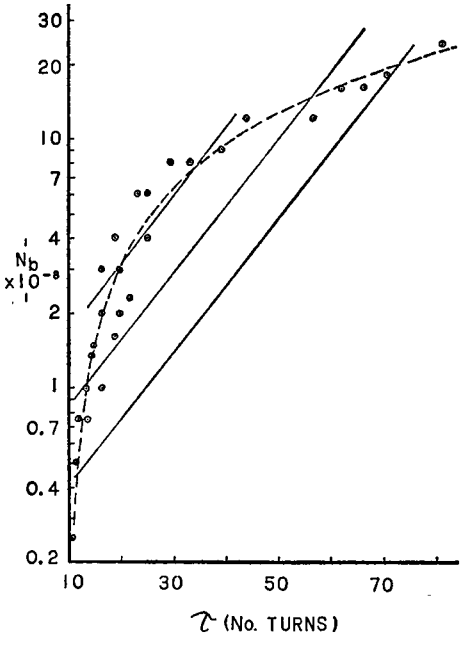
antibody concentrations represent a sequential increase in dilution from 1:500 to 1:16,000. It might be noted that the slopes of these lines ($dN_b/d\tau$) seem to be independent of antibody concentration. In an attempt to determine the validity of the assumption that the antibodies distribute evenly among the cells, dissociation rates were determined separately for 25, 33⅓, 50, 66⅔, and 75% deaggregation. If the bonds formed preferentially among certain cells in aggregate formation and if the aggregates were held together by these preferential bonds, then there should exist a critical degree of deaggregation beyond which the rate of deaggregation should rapidly increase (the aggregates would no longer be stable). Conversely, if most of these preferential bonds were located in the core of the aggregates, a sharp decrease in the rate of deaggregation would be anticipated. Fig. 11 represents data at three different temperatures for fraction deaggregation (expressed as N_b) vs. residence time (expressed as τ, number of turns) superimposed for the various degrees of bond breaking. It can be seen that the rate of bond breaking seems to be independent of the number of bonds broken and of the antibody concentration, thus validating the assumption of even distribution of antibody among the cells. Actually, a distribution curve may exist, but validation of the even distribution assumption necessitates that this curve be relatively steep.

DISCUSSION

If one attempts to interpret the results of these experiments in terms of simple chemical kinetic theory, rather interesting conclusions can be drawn. For example, Fig. 12 is a plot of log N_b vs. τ at 55.0°C and is representative of the various temperatures at which the system was studied. The straight solid lines in this figure are first-order kinetic equations plotted along with the data. The dotted curve, which provides a much better fit, is a zero-order kinetic equation. From the curve it must be noted that the system is apparent zero order following a model of the form $N_b = K\tau$. The zero-order rate constant K is, of course, temperature dependent but in a considerably more complex manner than is usually the case for simple homogeneous chemical reactions.

Fig. 13 shows a plot of log K vs. $1/T(°K)$, and is a consolidation of all the data in terms of the postulated zero-order rate constant. If the Arrhenius equation were followed this would be a straight line. Although it is not, the resulting curve can be resolved into two components, one of which is more important at higher temperatures.

It is easy to show that the aggregated cells are extremely sensitive to mechanical agitation, and it is likely that a portion of the bond breaking probably occurs by this mechanism. For example, it is easy to cause significant deaggregation of the aggregated cells simply by shaking the suspension in a test tube. If it is assumed that the deaggregation caused by agitation is temperature independent, then one can postulate that the horizontal asymptote at lower temperatures on the plot of log K vs.



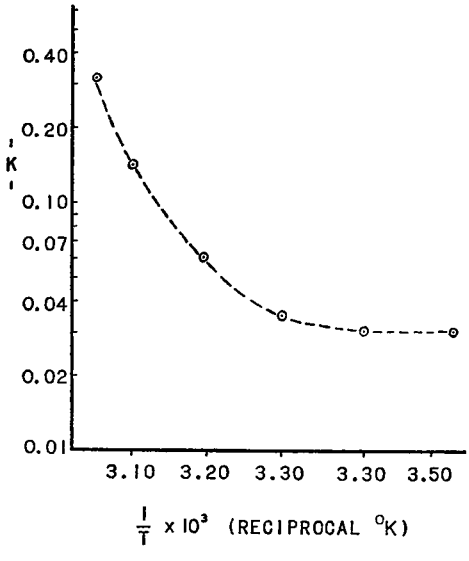


FIGURE 12

FIGURE 13

FIGURE 12 Semilogarithmic plot of number of bonds broken (N_b) vs. residence time (τ) at 55.0°C.

FIGURE 13 Arrhenius plot for postulated zero-order rate of bond breaking vs. temperature.

$1/T$ is the contribution to bond breaking by mechanical agitation. Thus, the over-all rate of bond breaking, $dN_b/d\tau$, can be thought to be made up of two separate processes: bond breaking due to mechanical agitation represented by a rate constant K_m, and bond breaking due to thermal kinetic effects represented by a rate constant K_t. If the value of the horizontal asymptote is taken to be equal to K_m and if this value is subtracted from the other K's, Fig. 14 results. Note that when log K_t is plotted against $1/T$, a straight line does result. From the slope of this straight line, the apparent activation energy of the reaction is readily calculated and is equal to 32.6 kcal/mole. This value of activation energy, while of the same order of magnitude as activation energies for a wide variety of typical organic bond-breaking reactions, is considerably higher than values reported for association of unbound anti-Rh_o and Rh_o positive erythrocytes by Hughes-Jones et al. (11).

Hughes-Jones et al. studied the association of ^{131}I-labeled Rh_o antibody with Rh_o positive erythrocytes and found the activation energy for this association reaction to be 13.5 kcal/mole. Since the heat of reaction for the equilibrium process was reported as 0.7 kcal/mole, the activation energy for dissociation of singly bound antibody molecules should therefore be of the order of 14.2 kcal/mole, as compared with 32.6 kcal/mole for the doubly bound antibody. Thus, it would appear that the

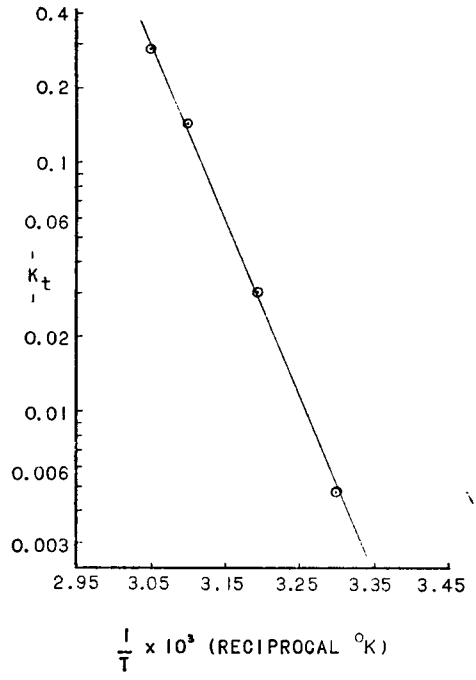


FIGURE 14 Plot of log K_t vs. the reciprocal of absolute temperature.

doubly bound red cell antibody system has more energetic bonds than does the singly bound antibody system.

At first glance, it might appear that an activation energy as high as 32.6 kcal/mole is incompatible with the observation that simple mechanical agitation can break these bonds; however, it must be remembered that red cells are considerably more massive than the molecules that one normally deals with in chemical reactions and, thus, would exert much more force on a bond in a shear field than would species in solution. In the case of bonds between species in solution, shear fields of the order of that which would be expected from simple mechanical agitation would not be expected to break bonds as strong as those represented by an activation energy of the order of 30 kcal/mole; but, of course, such species have inertia that is orders of magnitude less than that of red cells.

The most reasonable explanation for the difference in activation energy for the doubly bound vs. singly bound antibody lies in the fact that the systems are quite different. Since the red cells are extremely massive as compared with the antibody molecules, it is expected that the bonds in the doubly bound antibody system are stretched to a greater degree than in the singly bound system and therefore should be more "energetic." Furthermore, the Y-shaped γG antibody molecules may undergo additional stretching because of the flexibility of the hinge region about which the arms, each terminating in a combining site, can pivot. In addition, appar-

ent activation energy for the doubly bound antibody-antigen system may be actually a composite factor reflecting several energy-dependent processes which lead to the breaking of the antigen-antibody bond.

One such process might involve residual effects of Polybrene contributions to the aggregating forces after the addition of sodium citrate. Although the measurements were taken after the agglutinating effects of Polybrene would have been neutralized in the absence of an antibody, there were antibody molecules present in this system. Complete neutralization of Polybrene effects may occur more slowly in the presence of red cell aggregates held together by doubly bound antibody molecules because of slower diffusion of the citrate ion within the interstices of the aggregated cell mass. Although the role of Polybrene in the deaggregation process is unclear at the present time, it has been observed that characteristically different dissociation curves are obtained for different doubly bound antibody-antigen systems in the presence of Polybrene (12). This indicates that the deaggregation behavior reflects the nature of the particular antigen-antibody system being studied. Possible contributions of Polybrene to apparent activation energy are presently being explored.

The observation that the over-all bond-breaking reaction seems to be of apparent zero order requires some discussion. The rate of bond breaking seems to be independent of the number of antibody molecules added to the system and therefore independent of the number of antibody bridges present. The rate of bond breaking, therefore, would appear to be independent of the size of the aggregates as well. It is reasonable to assume that a three-dimensional red cell aggregate is a fairly stable structure. Thus, if bonds break in the interior of the aggregate, it seems reasonable that the red cells involved probably remain in place for a fairly long period of time. Thus, it is quite possible that the antigen sites remain within the region required for such a bond to reform, and the length of time that the red cells remain adjacent to each other may be essentially independent of the size of the aggregate. In the case of two aggregated red cells, it is likely that the red cells have enough inertia so that if a bond breaks, the cells still remain close enough for the bond to reform just as if they were present in a large aggregate.

In addition to the effects of electrostatic repulsive forces between red cells, which are presumably restored after the sodium citrate is added, the only processes available for moving the red cells out of range are brownian motion and convection. Convection obviously has a profound effect, since it provides the horizontal plateau region for the log K vs. $1/T$ plot (Fig. 13). That this convective effect is temperature independent indicates that the relaxation time for this process is quite long compared with the characteristic relaxation time for the kinetic process of bond breaking. These processes, therefore, should be essentially independent, and convection should affect large aggregates in exactly the same manner as it affects small ones.

At a given temperature, energy may be coupled into the antigen-antibody bond via direct "collisions" with energetic small molecules in solution or indirectly via vibration of the red cell membranes. It is most probable that red cell membranes

are vibrating continuously (because of thermal motion on the molecular level) and act as infinite reservoirs of energy to be supplied to the bonds. This might constitute the main source of energy input to the bonds of the doubly bound antibody molecule, as opposed to the singly bound molecule. Thus, the apparent zero-order kinetics might be a reflection of a constant rate of energy input into the bonds of doubly bound antibody molecules, regardless of the number of such molecules. It has yet to be determined over how large a range the system is zero order.

It is recognized that a single cell type (probable genotype R_1r) was used in this study. It is possible that different phenotypes might give different results; however, since the purpose of this work was to study one antigen-antibody type in detail, rather than to compare different types, no attempt was made to vary the source of either the cells or the antibody. The available evidence indicates that phenotype differences might not be very important in these studies. Experiments of the type discussed herein involving the *S* antigen and the Jk^a antigen have shown no difference between homozygous and heterozygous cells. Similarly, results of studies involving five different Rh_o antibodies have shown almost identical results with small differences which could be attributed to probable error in titration of the antibodies.[2]

CONCLUSION

A technique has been developed to study the chemical kinetics of doubly bound red cell antigen-antibody systems. In the case of anti-Rh_o antibody, the rate of dissociation of the antigen-antibody complex was calculated in terms of a rate of bond breaking and was found to follow apparent zero-order kinetics over the range that the data were obtained. It was found that the double binding of antibody between adjacent red cells apparently makes the bonds more energetic than would be the case for a singly bound antibody. The use of Polybrene may in part be responsible for this phenomenon.

In more recent work, antibodies other than Rh, such as those belonging to MNS and ABO systems, have been tested and have yielded similar results with rather different and characteristic apparent activation energies. This work will be reported in a separate communication.

The implications of these observations are important to the understanding of red cell antigen-antibody reactions from a physicochemical viewpoint. Physicochemical characterization of known red cell antigen-antibody systems may allow many in vivo effects associated with transfusion reactions to be interpreted in a new and more meaningful context. It is hoped that these methods will be applicable to unknown red cell antigen-antibody systems such as those responsible for red cell destruction in autoimmune diseases.

This work is based on a dissertation submitted by Bruce J. Oberhardt, in partial fulfillment of the requirements for the Ph.D. degree in Bioengineering at the Polytechnic Institute of Brooklyn.

[2] Oberhardt, B. J., P. Lalezari, and A. F. Jiang. Manuscript in preparation.

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