when most samples of phosphatidylserine (fraction III, Folch, 1942) are chromatographed on silicic acid-Hyflo Super-Cel columns.

2. Exchange of lipid sodium or potassium for another cation, presumably H^+ ion, occurs on the columns causing the elution of lipid with chloroform-methanol (4:1, v/v). This can be prevented by treatment of the adsorbent with hydrochloric acid followed by sodium hydrogen carbonate.

3. Exchange of lipid potassium for sodium occurs on the columns.

4. The chromatographic mobility of phosphatidylserine depends upon the metal ion combined with the lipid. Application of the calcium and magnesium forms to the column results in the elution of the lipid with chloroform-methanol (4:1, v/v). When the sodium or potassium forms are applied, the bulk of the lipid is eluted with chloroform-methanol (3:2, v/v).

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The Kinetics of the Reaction between the Blood-Group Antibody Anti-c and Erythrocytes

By N. C. HUGHES-JONES, BRIGITTE GARDNER AND RACHEL TELFORD

Medical Research Council's Experimental Haematology Research Unit, Wright-Fleming Institute of Microbiology, St Mary's Hospital Medical School, London, W. 2

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It has been realized for some time that antigen-antibody reactions are reversible, but it is only recently that precise physicochemical studies have been made and kinetic and thermodynamic constants reported. The subject has been reviewed by Talmage & Cann (1961).

The antibodies so far investigated have been mainly those produced in rabbits after the injection of various hapten-protein compounds. Only a few observations have been made on human red-cell antibodies. Filitti-Wurmser, Jacquot-Armand, Aubel-Lesure & Wurmser (1954) computed thermodynamic constants for anti-B antibody against group B erythrocytes; antibody concentration was measured indirectly, in terms of the maximum number of erythrocytes that the anti-serum could agglutinate. Boursnell, Coombs & Rizk (1953) used ^{131}I as a protein label to investigate the uptake by red cells of the antibody anti-D.

They found, however, that the red cells not only took up ^{131}I -labelled antibody but also considerable amounts of bound ^{131}I that was not associated with antibody, and which were large enough to mask the uptake of ^{131}I -labelled antibody. They termed this uptake of ^{131}I 'non-specific' and found that it was considerably decreased in the presence of unlabelled serum. Masouredis (1959) also used ^{131}I as a label for anti-D antibody. He overcame the problem of non-specific uptake by using antibody purified by elution from red cells. It has been shown that the non-specific uptake of ^{131}I by red cells from ^{131}I -labelled serum is due to three factors, namely, the absorption of ^{131}I -labelled serum proteins, the exchange of ^{131}I -labelled lipids between serum lipoproteins and red cells and the uptake of contaminating [^{131}I]iodide (Hughes-Jones & Gardner, 1962). Non-specific uptake of ^{131}I -labelled protein and lipid can be reduced by com-

petitive inhibition through the addition of unlabelled serum proteins; ^{131}I -labelled lipid and [^{131}I]iodide can be extracted with lipid solvents. In the experiments presented here, we have either employed these methods of reducing non-specific uptake of ^{131}I or have used purified antibodies in the investigation of the kinetics and thermodynamics of the reactions of an example of the red-cell antibody anti-c.

METHODS

Antiserum containing anti-c. The antiserum was obtained from a patient whose probable genotype in the Rh system was CDe/Cde and who had developed anti-c 2 years previously as a result of a blood transfusion and subsequent pregnancies. Only incomplete antibody was present and this appeared to be composed entirely of 7s γ -globulin (Dr M. Adinolfi, personal communication). The titre of anti-c was 2000. The serum also contained anti-A, which was removed by absorption before use.

Red cells. Human red cells were obtained from whole blood supplied by a blood bank. They were stored at 4° for 3–25 days before use. The red cells carrying the c antigen were of genotype cde/cde, and will be referred to as c-positive. The cells used as a negative control were of the probable genotype CDe/CDe, and will be referred to as c-negative. Only group O cells were used. The cells were washed four times with approximately 5–10 vol. of saline (buffered NaCl soln., see below) each time. After removing the supernatant of each wash, the top 10–20% of the packed cells was also sucked off to remove white cells and platelets. A cell suspension (haematocrit approx. 50%) was then centrifuged for 30 min. at 2000g in narrow tubes (0.5 cm. diam.) and only the cells from the lower half of the packed cell column were used for the experiments. The precise volume of cells used in the experiment was calculated from either the haematocrit or the haemoglobin concentration (a value for mean cell haemoglobin concentration of 34 g./100 ml. being used).

Iodination of antiserum. The serum was buffered to pH 8–9 with 1M-glycine-NaOH buffer. Carrier-free ^{131}I in weak thiosulphate solution was obtained from The Radiochemical Centre, Amersham, Bucks. Iodination was carried out by a modification of the method of Bournsnel *et al.* (1953). Free $^{131}\text{I}_2$ was obtained by adding [^{131}I]iodide to a solution of $^{127}\text{I}_2$ prepared by mixing 1 vol. of 25 mM-KI, 1.1 vol. of 5 mM-KIO₃ and 1 vol. of 0.1N-HCl; 0.1 ml. of this solution was used for every 35 mg. of serum protein. A portion (0.2 ml.) of 1M-KI was added to the serum 10 min. after the $^{131}\text{I}_2$, and excess of iodide removed by passing the serum through an anion-exchange column (Amberlite 401; Cl) (Veall, Pearson & Hanley, 1955). The ^{131}I -labelled serum was dialysed against 500 vol. of saline for 16 hr., and then centrifuged at 12 000g before use. The titre of anti-c in the final solution was usually 512–1024.

Specific activity of ^{131}I -labelled γ -globulin. As it was not possible to obtain purified antibody in a sufficient amount to determine the specific activity, it was assumed that the specific activity of the ^{131}I -labelled antibody was the same as that of the total γ -globulin. Stelos, Radzinski & Pressman (1962) have shown that the extent of the iodination of papain fractions of purified rabbit antibody differs

from that of normal γ -globulin; hence the assumption about the specific activity of ^{131}I -labelled antibody may be incorrect and introduce an error in the determination of antibody concentration. γ -Globulin was prepared from the ^{131}I -labelled antiserum with 6,9-diamino-2-ethoxyacridine lactate according to the method of Hořejší & Smetana (1956). The ^{131}I and protein content were then each estimated in duplicate. The iodinated protein usually contained about 1 atom of iodine, and never more than 2 atoms, per molecule of protein. The molar concentration of the antibody solutions could then be calculated from the specific activity of γ -globulin, assuming a molecular weight of 160 000.

Protein determination. This was by the method of Folin & Ciocalteu, as modified by Kunkel & Tiselius (1951), γ -globulin being used as a standard.

^{131}I content. This was estimated with a well-type scintillation counter and a pulse-height analyser. A total count of 2500 was usually made, except when the counting rate was low (i.e. 50 counts/min. or less), when at least 1000 counts were made. The background counts were approx. 25 counts/min.

Extraction with ether-ethanol. Those cells and antibody solutions that were to be extracted were first freeze-dried. If the total protein content of the antibody solutions was low, unlabelled carrier protein was added before drying. The dried residue was then extracted twice with 5 ml. of ether-ethanol (1:3, v/v), 1 hr. being allowed for each extraction. Ether-ethanol extracted ^{131}I -labelled lipids and [^{131}I]iodide. The fall in ^{131}I content of the c-positive red cells after extraction varied from nil (i.e. no detectable change outside experimental error) to 50% of the total ^{131}I on the cells.

Saline. 0.17M-Sodium chloride solution has been used throughout, buffered with Na₂HPO₄-NaH₂PO₄ solution, 0.01M, pH 6.7.

Unlabelled serum proteins. Serum was obtained from donors of blood group A, and concentrated by pressure-dialysis against saline.

Antibody titre. The antibody titre of the serum has been determined with antiglobulin serum by conventional methods (Mollison, 1961).

Purification of anti-c by dissociation of the antibody-antigen complex

The purification of ^{131}I -labelled anti-c was based on the supposition that the antigen-antibody reaction is reversible and obeys the laws of mass action. Therefore, on placing red cells containing antigen-antibody complex in saline, the complex will dissociate to bring about equilibrium concentrations. Thus cells were suspended in 20–40 ml. of saline and dissociation was allowed to proceed for 30 min. at 37°. After centrifuging and removal of the supernatant the dissociation was repeated as many times as required.

^{131}I -Labelled serum containing anti-c was diluted with 4 vol. of saline containing unlabelled serum proteins in a concentration of 10 g./100 ml. (final ratio of labelled to unlabelled protein being approx. 1:10–1:20). The purpose of the unlabelled proteins was to diminish the non-specific uptake of ^{131}I -labelled protein. The final antibody titre was usually either 128 or 256. A portion (10 vol.) of this diluted ^{131}I -labelled serum was added to 1 vol. of packed red cells at 37° for 30 min. (sufficient for equilibrium concentrations

almost to be reached, see Results section). The red-cell suspension was then centrifuged at 2000g and the supernatant removed. The cells were washed eight times in saline at 0° (kept cool to decrease the dissociation of the complex). This washing procedure has been shown to decrease the amount of ^{131}I -labelled serum trapped in the red-cell deposit to between 1×10^{-6} and 1×10^{-7} of its original value, which was never more than the equivalent of 1×10^7 counts/min. The red cells were then suspended in 20–40 vol. of saline at 37° for 30 min. to allow the complex to dissociate (this step will hereafter be referred to as an elution). After centrifuging and removal of the supernatant, 10 mg. of serum proteins was added to reduce the loss of antibody through adsorption on to the walls of the containers. The elution could be repeated as many times as required. The eluted antibody was then concentrated by pressure-dialysis. The amount of free antibody that can be obtained in this way is shown in Fig. 1. In this example, the cells were suspended in 20 vol. of saline on each occasion. 60% of the antibody was removed by five elutions and 80% by 10 elutions. For the eleventh elution, the cells were suspended in 200 vol. and a further 15% was removed.

As serum proteins are added to the eluted antibody to diminish loss through absorption, the purification is only true in the sense that ^{131}I -labelled antibody is separated from other ^{131}I -labelled compounds. The extent of this isolation has been investigated and it has been found that at least 90–95% of the ^{131}I in the eluates represents antibody that can be absorbed by c-positive red cells. Before these eluates were used, unlabelled serum proteins were added to give a final concentration of 5–10 g./100 ml. Under these conditions, it was found that the uptake of ^{131}I by the c-negative cells was only 2% or less of that taken up by the c-positive cells under the same conditions. As the non-specific uptake was so low, extraction of the c-positive cells and the antibody with ether-ethanol was not necessary.

Rate of association between antibody and antigen at 19° and 37°, with purified ^{131}I -labelled anti-c

For this experiment, purified anti-c obtained from the first four elutions (see preceding section) was diluted to give a final volume of 50 ml. The final concentration of antibody was 2.5 μM . The antibody solution was added to 1.6 ml. of c-positive packed red cells and 25 ml. was incu-

bated at 37° and 25 ml. at 19°; portions (3 ml.) were removed from both suspensions at appropriate intervals and the reaction was immediately stopped by centrifuging at 2000g for 5 min. The supernatant containing the free antibody was removed as completely as possible, the cells were cooled to 0°, 1 ml. of saline at 0° was added and the suspension immediately centrifuged again. This wash was carried out at 0° to decrease the dissociation of the complex to the minimum value. Subsidiary experiments showed that not more than 1–2% of the complex dissociated under these washing conditions. After separation of the cells from the supernatant, the ^{131}I content was estimated.

In determining *t* (the time during which reaction has proceeded), it is clear that a precise time for stopping the reaction by centrifuging cannot be given; however, it was found that separation was complete within 20 sec. from turning on the centrifuge, and this value was taken into account. This applies to all other experiments where centrifuging was used for stopping the reaction.

Rate of dissociation of the antigen-antibody complex at 37°

^{131}I -Labelled serum containing anti-c (titre 256) was diluted eightfold with saline containing unlabelled serum proteins at a concentration of 10 g./100 ml. A portion (10 ml.) of this diluted ^{131}I -labelled serum (titre 32) was added to 1 ml. of c-positive packed red cells and left at 37° for 30 min. The cell suspension was then centrifuged, the supernatant removed and the cells were washed eight times with saline at 0°. The cells were then added to 40 ml. of saline at 37° to dissociate the complex; samples (5 ml.) were removed at appropriate intervals and the dissociation was stopped immediately by centrifuging. The cells were cooled and washed at 0° with 1 ml. of saline. The cells and supernatant were extracted with ether-ethanol and the ^{131}I content was determined. The same experiment was carried out with c-negative cells as controls. The ^{131}I content of the control cells and their corresponding supernatants were between 5 and 10% of that on the c-positive cells, and these values were subtracted from those of the c-positive cells.

Determination of the equilibrium concentrations for the antigen-antibody reactions, at different antibody concentrations

With ^{131}I -labelled serum containing anti-c. This experiment involved two procedures. The first procedure was to add varying concentrations (titres ranging from 256 to 8) of anti-c to red cells and determine the uptake of ^{131}I -labelled antibody at equilibrium for each antibody concentration. Secondly, the total antibody content of the serum was determined by repeated absorption of the serum with c-positive red cells. ^{131}I -Labelled antiserum (titre 1024) was diluted fourfold with saline containing 10 g. of unlabelled serum proteins/100 ml. and centrifuged at 12 000g for 20 min. Serial doubling dilutions (i.e. 1:2, 1:4 etc.) were made from this diluted antiserum. A portion (2 ml.) of each dilution was then added to 0.2 ml. of c-positive packed cells and incubated at 37° for 60 min. with frequent mixing. This is sufficient time for equilibrium concentrations to be reached (see Results section). The suspensions were then centrifuged, the supernatants removed and the

Fig. 1. Extent of the removal of antibody bound to red cells by 10 successive elutions into 20 vol. of saline. The eleventh elution was carried out in 200 vol. of saline. Ordinate: moles of antibody remaining bound to 1 ml. of red cells after each elution.

cells washed eight times with saline at room temperature. The cells were then extracted with ether-ethanol and the remaining ^{125}I content was estimated. The same procedure was also carried out with c-negative cells for the determination of non-specific uptake of ^{125}I at each dilution. Values for the controls were subtracted from values for the c-positive cells. Uptake by c-negative cells ranged from 3% of that of the c-positive cells at the highest antibody concentrations to 20% at the lowest antibody concentrations.

Determination of the total antibody content of the ^{125}I -labelled antiserum was made by repeatedly absorbing samples of serum with 0.2 ml. of c-positive packed red cells at 37° for 30 min. When the red-cell uptake of ^{125}I with each absorption was plotted, the curve was found to be asymptotic. Thus after five or six absorptions the ^{125}I uptake had reached a low and almost constant value at about 10% of that of the first absorption; the lowest value found was taken to represent the amount of non-specific ^{125}I in all the absorptions. It is possible that the uptake of non-specific ^{125}I was higher than this value in the initial absorptions; if this were so, then the antibody content has been overestimated by this method. The average of the values obtained by absorbing three samples of the serum were used. With these data, the antibody content of the solutions added to the cells in the first half of the experiment could be calculated. The concentration of free antibody at equilibrium was calculated as the difference between the total amount of antibody added and that taken up by the red cells.

With purified ^{125}I -labelled antibody. The isolation of the ^{125}I -labelled antibody was carried out according to the method described above. The ^{125}I -labelled antiserum was diluted fourfold with saline containing 10 g. of unlabelled proteins/100 ml. and was absorbed twice with 0.1 vol. of c-positive cells. Four successive elutions of antibody were carried out from the cells used for the first absorption and twice from the cells used for the second absorption. All the eluates were concentrated and pooled. A series of doubling dilutions of the purified antibody were made with saline containing 5 g. of unlabelled serum proteins/100 ml. A portion (1 ml.) of each dilution was added to 20 μl . of c-positive cells. Control experiments with c-negative cells were also carried out. The suspensions were kept at 37° for 1 hr. with frequent mixing and then centrifuged, the supernatants removed, the cells washed once with 1 ml. of saline at 0° , and ^{125}I content was estimated in the cells and in the suspending solution.

In a second experiment with purified antibody, eluates were prepared in the same way, but they were not pooled, each eluate being concentrated separately and used separately for the determination of the association constant; the final volume was adjusted so that they all contained the same concentration of antibody. A portion (3.0 ml.) of each eluate was added to 0.1 ml. of c-positive cells; c-negative cells were used as controls. The subsequent procedure was the same as in the previous experiment.

The uptake of ^{125}I by the control cells in these experiments was only 2% of that of the c-positive cells. As this value was so low, no extractions with ether-ethanol were carried out and no correction for non-specific uptake was made.

Determination of the association constant at 15° and 40° . Ten samples of red-cell suspensions (c-positive) were made

with 0.19 ml. of c-positive cells in 3 ml. of saline containing purified ^{125}I -labelled antibody (3.5 μM). Five samples of the suspension were placed at 15° for 300 min. and five samples at 40° for 60 min., with frequent mixing. The cells were centrifuged, the supernatants removed and the cells washed once with 1 ml. of saline at 0° . The ^{125}I content of the cells and supernatant was determined. The antibody was also added to c-negative cells at each temperature. ^{125}I counts on the control cells were approximately 2% of those on the c-positive cells; no extractions were carried out.

Calculations

The association constant K was calculated according to the equation

$$\frac{[\text{Ag Ab}]}{[\text{Ag}] \times [\text{Ab}]} = \frac{k_1}{k_2} = K \text{ l.mole}^{-1} \quad (1)$$

where $[\text{Ag Ab}]$, $[\text{Ag}]$ and $[\text{Ab}]$ are the molar equilibrium concentrations of the complex, antigen and antibody respectively. The molecular weight of antibody was taken to be 160 000. As shown below, the antigen-antibody reaction is in fact heterogeneous with respect to its intrinsic binding constant; the calculated values of K therefore only indicate the order of magnitude, the actual range of values extending above and below the calculated figure.

The antigen concentration was estimated indirectly from the determination of the maximum uptake of antibody by the red cells, this latter being estimated to be 1.2×10^{-9} moles/ml. of red cells (see Results section). For the purpose of the calculations, it was assumed that only one binding site on the antibody molecule is involved in the reaction and that each antigen-binding site represents 1 molecule of antigen. All calculations of the association constant therefore assumed that the red-cell-antigen content is 1.2×10^{-9} moles/ml. of red cells.

Equation for the rate of association of antigen and antibody.

$$k_1(a-x)(b-x) = k_2x$$

where k_1 and k_2 are rate constants; a is the initial antigen concentration; b is the initial antibody concentration; x is the concentration of antigen-antibody complex.

$$\text{Then} \quad \frac{dx}{dt} = k_1(a-x)(b-x) - k_2x$$

$$\text{solving} \quad \ln \left(\frac{Z+A}{Z-A} \right) = -2Ak_1t + B \quad (2)$$

$$\text{where} \quad Z = x - \left(\frac{a+b+c}{2} \right)$$

$$c = \frac{k_2}{k_1}$$

$$A^2 = \left(\frac{a+b+c}{2} \right)^2 - ab$$

$$B = \ln \left(\frac{Z_0 + A}{Z_0 - A} \right) \text{ when } Z_0 = - \left(\frac{a+b+c}{2} \right)$$

Equation for rate of dissociation of antigen-antibody complex.

$$k_2(c-x) = k_1(a+x)(b+x)$$

where k_1 and k_2 are rate constants; a and b are the antigen and antibody concentrations; c is the antigen-antibody

complex concentration; x is the concentration of complex dissociated at any time t .

$$\text{Then} \quad \frac{dx}{dt} = k_2(c-x) - k_1(a+x)(b+x)$$

$$\text{solving} \quad k_2 t = \frac{\ln \left[\left(\frac{x-v}{x-u} \right) \left(\frac{u}{v} \right) \right]}{\sqrt{\{4K(c-Kab) + [1+K(a+b)]^2\}}} \quad (3)$$

$$\text{where} \quad u = -\frac{R}{2} + \sqrt{\left(S + \frac{R^2}{4}\right)}$$

$$v = -\frac{R}{2} - \sqrt{\left(S + \frac{R^2}{4}\right)}$$

$$R = \frac{1}{K} + a + b$$

$$S = \frac{c}{K} - ab$$

$$K = \frac{k_1}{k_2} = \frac{(c-x_e)}{(a+x_e)(b+x_e)} \text{ at equilibrium.}$$

RESULTS

Rate of association between antibody and antigen at 19° and 37°, with purified ¹³¹I-labelled anti-c

The rates of formation of the antibody-antigen complex at 19° and 37° are given in Fig. 2. The association between antigen and antibody to form antibody-antigen complex is not represented by the equation for straightforward second-order reactions, as the rate of reaction is affected by the simultaneous dissociation of the formed complex. The equation representing the rate of formation of the complex is given in the previous section (equation 2). The rate constant, k_1 , for the association reaction can be calculated by substituting the observed values obtained from this experiment into equation (2).

At 37°, the values of k_1 obtained from the first five observed values (2–32 min.) decreased successively from 5.3×10^4 to 2.1×10^4 l.mole⁻¹ sec.⁻¹ (average 3.2×10^4). Similarly, the first six observed values (4–120 min.) at 19° gave values of k_1 descending from 9.7×10^3 to 3.2×10^3 l.mole⁻¹ sec.⁻¹ (average 4.6×10^3). In Fig. 2 the continuous lines are the calculated curves (equation 2) for the rate of formation of the complex when the average values of k_1 are used. Because of the variation in the calculated values of k_1 , the initial observed values are higher than the curve and later values are lower. The association constants calculated from the equilibrium values in this experiment were K 1.9×10^7 l.mole⁻¹ at 37°, and K 2.2×10^7 l.mole⁻¹ at 19°.

Rate of dissociation of the antigen-antibody complex at 37°

Two experiments were carried out on the determination of the rate of dissociation of the complex.

The results of one of these experiments is given in Fig. 3. The other experiment gave similar results. The dissociation of the complex should be a unimolecular reaction modified by the subsequent recombination of antigen and antibody. The mathematical representation of this type of curve has been given in the previous section. Substituting observed values in equation (3), values for the rate constant k_2 are obtained, which decrease progressively from 6.8×10^{-4} to 2.6×10^{-4} sec.⁻¹ in one case, and from 5.2×10^{-4} to 1.1×10^{-4} sec.⁻¹ in the other case, as t increases. The calculated curve for the rate of dissociation is given by the continuous

Fig. 2. Rate of formation of antigen-antibody complex at 19° (○) and 37° (●). Continuous curves are derived from equation (2), average values being used for the rate constant for association, k_1 .

Fig. 3. Example of the rate of dissociation of the antigen-antibody complex. ○, Concentration of antigen-antibody complex; ●, concentration of free antibody. Continuous curves are derived from equation (3), an average value being used for the rate constant for dissociation, k_2 .

lines in Fig. 3, the average value of k_2 being used in each experiment. The observed values do not fit the curve, the initial observed concentrations of the complex being lower and the later observations higher than the calculated curve. The association constants calculated from the data of these experiments were $K\ 1.8 \times 10^8$, and $K\ 1.2 \times 10^8\ \text{l.mole}^{-1}$. These equilibrium constants are higher than those found in the previous section; this is probably a reflexion of the different methods of preparing the antibody.

Determination of the equilibrium concentrations for the antigen-antibody reaction at different antibody concentrations

With ^{131}I -labelled serum containing anti-c. Two experiments were carried out with ^{131}I -labelled serum containing anti-c. In one experiment, the iodinated antiserum giving a titre of 256 contained $21\ \mu\text{g.}$ of antibody/ml. ($0.13\ \mu\text{M}$); in the other experiment, a titre of 128 corresponded to an antibody content of $14\ \mu\text{g./ml.}$ ($0.087\ \mu\text{M}$). Scatchard (1949) has discussed the use of derivations of the law of mass action which can be used for the graphical representation of equilibrium values; these curves can be used to determine the maximum uptake of antibody by the red cells. The equilibrium values have been used to obtain a curve according to the following equation of Scatchard:

$$r/[A] = Kn - Kr \quad (4)$$

where r is the amount of antibody taken up by the red cells, n , the maximum number of antibody molecules that can be bound on the red cells, $[A]$, the concentration of free antibody, and K , the association constant. Plotting $r/[A]$ against r will give a straight line only if the antibody is homogeneous with respect to K . Extrapolation of the curve to zero value of $r/[A]$ will give n . Fig. 4 gives these relationships in the two experiments. The curve is curvilinear, which indicates that the antibody-antigen reaction is not homogeneous with respect to K . A precise extrapolation of the curve to a value of $r/[A] = 0$ is difficult; a value of 1.2×10^{-9} moles/ml. of red cells has been taken as the maximum uptake of antibody.

With purified ^{131}I -labelled antibody. The determination of equilibrium values for r and $[A]$ has also been done with purified antibody. In the first experiment, six eluates of antibody were obtained and all pooled and concentrated. Serial dilutions of the antibody were then made. The results obtained from this experiment have been plotted according to equation (4) in Fig. 5. The shape of the curve indicates heterogeneity of the intrinsic binding constant. If there is heterogeneity of the association constant and if this is due mainly to

variation in the rate constant for dissociation, k_2 , then the antibody with a lower intrinsic binding constant will dissociate more rapidly from the red cells than antibody with a higher binding constant. Thus each successive dissociation of antibody from red cells should contain antibody with a progressively higher range of intrinsic binding constants. Therefore, in a second experiment with purified anti-c, six successive eluates were prepared and concentrated separately. The association constants for the antibody in each successive eluate were as follows, starting with the eluate first obtained from the red cells: 2.2, 2.7, 6.1, 7.3, 7.8 and $8.4 \times 10^7\ \text{l.mole}^{-1}$. Each successive eluate therefore contains antibody with a progressively higher range of association constants.

Determination of the association constant at 15° and 40°. The association constants K determined at 15° and 40° were $2.07 \pm 0.11 \times 10^7\ \text{l.mole}^{-1}$ at 15° and $1.69 \pm 0.04 \times 10^7\ \text{l.mole}^{-1}$ at 40°.

Fig. 4. Equilibrium relationships between the amount of antibody on the red cells, r (10^{-10} moles/ml.), and the ratio $r/[A]$, where $[A]$ is the molar concentration of free antibody. Source of antibody was whole serum containing anti-c. The results were obtained from two experiments, ● and ○.

Fig. 5. Equilibrium relationships between the amount of antibody on the red cells, r (10^{-10} moles/ml.), and the ratio $r/[A]$, where $[A]$ is the molar concentration of free antibody. Source of antibody was purified anti-c.

DISCUSSION

In these experiments, the non-specific uptake of ^{131}I from ^{131}I -labelled antiserum has been reduced either by adding high concentrations of unlabelled serum proteins to the iodinated antiserum or in some cases by extraction of the cells with ether-ethanol. The amount of ^{131}I extracted by ether-ethanol has varied considerably in different experiments; the cause of this variation is not known. Use of whole antiserum, however, has the disadvantage that the concentration of ^{131}I -labelled antibody is only of the order of one-thousandth part or less of the total ^{131}I -labelled proteins; hence in the separation of cells from the suspending solution by centrifuging, trapping of 'non-antibody' protein in the packed red cells masks the antibody absorbed by the cells. The cells must therefore be washed, but in doing this some of the antibody on the cells will dissociate from the antigen-antibody complex. We have found that when cells are washed in saline at a temperature between 0° and 10° , approximately 1-2% of the anti-c dissociates with each wash. In the experiments in which the cells were washed at a temperature between 15° and 20° , the loss must have been greater with each wash. The results therefore underestimate the amount of antibody combined with the red cells under equilibrium conditions. Moreover, in the experiments with ^{131}I -labelled antiserum, the antibody content of the antiserum has to be determined by the repeated absorption of antibody by red cells. The free antibody concentration at equilibrium is then calculated as the difference between the amount of antibody added initially and the amount absorbed by the red cells. Small errors in the estimation of the antibody content of the serum and in the preparation of the doubling dilutions lead to large errors in the estimates of free antibody concentration, especially when that concentration is low. Thus although in the experiments with whole antiserum the results suggest that the antibody-antigen reaction is heterogeneous with respect to the intrinsic binding constant (Fig. 4) it is possible that this could have been due to experimental error.

The results obtained with the purified ^{131}I -labelled anti-c are considerably more accurate. The non-specific uptake of ^{131}I by c-negative cells from the purified antibody solutions was not more than 2%, and the absorption experiments have shown that at least 90% of the ^{131}I represents ^{131}I -labelled antibody. With antibody pooled from six successive elutions of red cells, the plot of r against $r/[A]$ (Fig. 5) showed that the antibody-antigen reaction was heterogeneous with respect to K .

Further evidence of the heterogeneity of the association constant has been obtained by concen-

trating separately each successive elution of antibody from the red cells. With these individual eluates with the narrow range of equilibrium constants, association constants ranging from 2.2×10^7 to $8.4 \times 10^7 \text{ l.mole}^{-1}$ were obtained.

The purified antibody used in this latter experiment was approximately the first 60% that had dissociated from the antibody-antigen complex, and hence would be expected to consist mainly of the antibody with the lower intrinsic binding constant. Antibody with the higher binding constant can best be demonstrated by adding to red cells whole serum containing antibody diluted to a low concentration (titre of 32). At low concentrations of free antibody, the antibody-antigen complex that is formed is composed mainly of associations with the highest binding affinity. After washing of the cells, the complex is allowed to dissociate. This is illustrated in the experiment on the determination of the rate of dissociation of the antibody-antigen complex (Fig. 3); association constants of 1.2×10^8 and $1.8 \times 10^8 \text{ l.mole}^{-1}$ were found in these two examples. In these two experiments we have thus found a range of the association constant between 2.2×10^7 and $1.8 \times 10^8 \text{ l.mole}^{-1}$. The lowest association constant recorded was $1.9 \times 10^7 \text{ l.mole}^{-1}$ found in one of the experiments on the determination of the rate of association. It is probable that all the antibody preparations were heterogeneous, and hence the true range undoubtedly extends on either side of the observed values.

The calculation of the equilibrium constants involves an accurate assessment of the total antigen content of the red cells. The method of assessment of the value used in these experiments is open to three sources of error. In the first place, an estimation of the maximum uptake of antibody depends on extrapolating a curve a considerable distance (Fig. 4), which must necessarily be inaccurate. Secondly, some antibody was lost from the cells when they were washed, and hence the maximum antibody-binding capacity was underestimated. Thirdly, the number of antigen sites which combine with a molecule of antibody is at present not known. All antibodies that have been fully investigated have been found to have two combining sites, and it is possible that the spatial distribution of the antigen sites allows some or all of the antibody molecules to combine with both their combining sites. Until this relationship is known, we have assumed for the purpose of calculation that one antibody molecule is attached to one antigen site only. Estimates of the total number of antigen sites, and hence the association constants, may therefore be in error by a factor of 2. With these reservations, we have estimated the antigen concentration of c-positive (cc) red cells to be

1.2×10^{-9} moles/ml. If the volume of a single red cell is taken to be $90\mu^3$, then there are approximately 65 000 antigen sites/red cell.

It has been assumed that the association reaction is a bimolecular reaction and that the dissociation reaction is unimolecular; the kinetics of both are complicated by the fact that the reactions do not go to completion but come into equilibrium. In the derivation of equations (2) and (3), which represent these reactions, two assumptions have been made, namely, that the antibody under investigation is homogeneous with respect to its rate constants, and that there is no interaction between antigen-antibody complexes. The observed values do not fit the calculated curves for the rates of association and dissociation if single values for the rate constants are used. A reasonable explanation of the discrepancy would be that the antibody is heterogeneous with respect to the rate constants. Equations (2) and (3) are not designed to measure the degree of heterogeneity of the rate constants, and thus the calculated values of k_1 and k_2 do not represent the true range of the constants. An alternative explanation of the discrepancy is that there is an interaction between antigen-antibody complexes and that the negative charge on the antibody molecule of the complex repels free antibody molecules from neighbouring antigen sites. This is perhaps unlikely in the experiments shown here, where only approximately 3 % of the total antigen sites are occupied.

A third possibility is that the basic assumptions are incorrect and that the association and dissociation reactions are not bimolecular and unimolecular respectively. Thus Talmage (1960) suggested that the reaction between albumin and anti-albumin antibody takes place in two stages, that is, after the initial association there is a slow recombination and rearrangement so that the final complex has a higher intrinsic association constant than the initial complex. Lafferty & Oertelis (1961) have also suggested a two-phase reaction for anti-viral antibodies.

The experiments on the rate of association were carried out with antibody preparations possessing the lower intrinsic binding constants; K and k_1 were determined experimentally, and k_2 can then be calculated from the relationship $k_1/k_2 = K$ (the average value for k_1 being used). Similarly, the experiments on the rate of dissociation were carried out on antibody preparations possessing the higher intrinsic binding constants; K , k_1 and k_2 can be similarly estimated. These values are given in Table 1. Although these results are taken from only three separate experiments, it appears that the difference between the antibody of high and low binding capacity is due mainly to the difference in the rates of dissociation, k_2 , whereas there is less difference between the rates of association, k_1 .

For the lowest and highest observed values for K obtained at 37° , the standard free energy change can be calculated from the equation

$$\Delta F_0 = -4.575 T \log K$$

to be -10.1 and -11.3 kcal./mole. These values are of the same order as those obtained for the reaction between the antigens insulin (Berson & Yalow, 1958), ϵ -*N*-dinitrophenyl-lysine (Velick, Parker & Eisen, 1960) and fluorescein-ovalbumin (Dandliker & Feigen, 1961) and their respective antibodies.

The equilibrium constant has been determined at 15° and 40° and it has been found that K decreases from $2.01 \pm 0.04 \times 10^7$ l.mole $^{-1}$ at 15° to $1.67 \pm 0.1 \times 10^7$ l.mole $^{-1}$ at 40° . The standard deviation of the results refers only to the errors arising from the pipetting of solutions and the estimation of ^{131}I content, and does not take into account the inaccuracy in the determination of the antigen content of the red cells. Any errors in the estimation of antigen content, however, will not affect the ratio of the association constants at the two temperatures. The standard enthalpy change can be determined from these values; a value of

$$\Delta H_0 = -1.5 \pm 0.6 \text{ kcal./mole}$$

was obtained. With this value, and the lower value of -10.1 kcal./mole for ΔF_0 , the standard change in entropy is $\Delta S_0 = 28$ cal./degree/mole. The change in free energy of the association reaction is therefore mainly associated with a change in entropy.

Although the intrinsic binding constant for anti-c is high, the antibody-antigen complex can be dissociated by repeatedly suspending the cells in large volumes of saline; the eluted antibody can be concentrated by pressure-dialysis. This process has been used as a simple method for the purification of the antibody with a high yield and can be applied to other antibodies with similar intrinsic binding constants.

Table 1. *Estimated values of K , k_1 and k_2 at 37° for two different antibody preparations*

	Antibody preparations with high association constant	Antibody preparation with low association constant
K (l.mole $^{-1}$)	1.8×10^8 1.2×10^8	1.9×10^7
k_1 (l.mole $^{-1}$ sec. $^{-1}$)	7.6×10^4 2.6×10^4	3.2×10^4
k_2 (sec. $^{-1}$)	4.2×10^{-4} 2.2×10^{-4}	1.7×10^{-3}

SUMMARY

1. The kinetics of the association between an example of the red-cell antibody anti-c and the corresponding antigen on the red cell were investigated.

2. The non-specific uptake of ^{131}I by red cells from iodinated antiserum was reduced by competitive inhibition with unlabelled serum proteins; further reduction was made by extraction of the red cells with lipid solvents, which removes ^{131}I -labelled lipids and ^{131}I iodide.

3. Two estimates were made of the relationship between antibody titre and antibody concentration; thus a titre of 256 corresponded to a concentration of $0.13\ \mu\text{M}$ ($21\ \mu\text{g./ml.}$) and a titre of 128 corresponded to a concentration of $0.087\ \mu\text{M}$ ($14\ \mu\text{g./ml.}$).

4. The number of c antigen sites on each red cell was estimated to be 65 000.

5. The reaction between antibody and antigen was reversible and heterogeneous with respect to its equilibrium constant. The minimum range of the association constants was

$$K\ 1.9 \times 10^7 - 1.8 \times 10^8\ \text{l.mole}^{-1}$$

6. The values of the thermodynamic constants were: standard free energy change, $\Delta F_0 = -10.1 - 11.3\ \text{kcal./mole}$; standard enthalpy change, $\Delta H_0 = -1.5 \pm 0.6\ \text{kcal./mole}$; standard entropy change, $\Delta S_0 = 28\ \text{cal./degree/mole}$.

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The Measurement of Entry Rates of Propionate and of Butyrate in Sheep

By E. F. ANNISON AND D. B. LINDSAY

School of Rural Science, University of New England, Armidale, N.S.W., Australia

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The importance of volatile fatty acids as major sources of energy in ruminant metabolism is now well established. Acetic acid, propionic acid and butyric acid, together with small amounts of acids of chain length C_6 - C_7 , are produced by the ruminal fermentation of carbohydrate and protein. The absorption of these acids from sheep rumen was first demonstrated by Barcroft, McAnally & Phillipson (1944), but quantitative assessment of the amounts of the various acids absorbed has

proved difficult. Methods based on the comparison of fatty acid production in samples of rumen contents incubated *in vitro* with the production *in vivo* (Carroll & Hungate, 1954; Halse & Velle, 1956), or on the analysis of portal blood (Schambye, 1951; Annison, Hill & Lewis, 1957), are subject to several major disadvantages (Annison & Lewis, 1959). A more promising approach is the use of isotope dilution, which has been applied to the measurement of the entry rates of acetate and glucose in