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Studies on the Reaction between the Blood-Group Antibody Anti-D and Erythrocytes

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Radioactive iodine (^{131}I) was first used as a label for the blood-group antibody anti-D by Boursnell, Coombs & Rizk (1953). These authors estimated the antibody content of a particular example of anti-D serum to be 54 μg./ml. and also estimated the total number of antigen sites on Rh-positive cells (probable genotype CDe/CDe) to be 5500/red cell. Masouredis (1959) carried out similar experiments and estimated the number of antigenic sites to be between 2000 and 3000/red cell. In later observations, Masouredis (1960*b*) estimated that cells homozygous for the antigen D had either 10 300 or 7400 antigen sites/red cell, depending on the absence or presence respectively of the blood-group antigen C. Masouredis (1960*a*) also estimated the extent of the transfer of ^{131}I-labelled anti-D antibody from sensitized red cells to unsensitized cells. For one particular example of anti-D he found that approx. 15% was transferred in 1 hr. and thereafter no further transfer could be demonstrated. Evans, Mebust & Hickey (1960), using conventional techniques with antiglobulin sera, found that the extent and rate of the transfer of anti-D between red cells was consistent with the law of mass action and they confirmed their results by using ^{131}I-labelled anti-D.

Hughes-Jones, Gardner & Telford (1962) presented the results of investigations on the kinetics of the reaction between the human blood-group antibody anti-c and erythrocytes by using ^{131}I as a label for the antibody. An analysis of the law of mass action first suggested by Scatchard (1949) was used to demonstrate heterogeneity of the intrinsic binding constant between antigen and antibody and also to determine the number of antigen sites on the red cell. Similar investigations on three examples of the human blood-group antibody anti-D are the subject of the present paper.

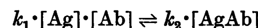
METHODS

Antibody-containing sera. Three sera were investigated: Av, Ke and Wa. In each case the antibody was an incomplete anti-D, each known to have the characteristics of a 7s γ-globulin. Serum Av had a titre of 1000 (indirect anti-globulin method) and was obtained 3 months after the injection of Rh-positive red cells that were given to re-stimulate antibody production. γ-Globulin was prepared by ether fractionation from sera Ke and Wa by Dr R. Kekwick of the Lister Institute; these γ-globulin preparations had titres of 1000 and 500 respectively.

Red cells. All the red cells used were of the phenotype CCDee; they were probably (P 0·9) of the genotype CDe/CDe and are referred to below as R_1R_1. The control cells were of the genotype cde/cde (referred to as rr cells).

Labelling of γ-globulin solutions with ^{131}I and determination of the specific activity. These were done by the methods described by Hughes-Jones *et al.* (1962). γ-Globulin was prepared from serum Av by precipitation with 1·8M-ammonium sulphate before labelling. The uptake of iodine by γ-globulin was usually between 1 and 2 atoms of iodine/molecule of protein.

Calculations. The rate constants for association and dissociation were calculated on the assumption that the reversible reaction of antigen and antibody could be represented by:



$$k_1\cdot[\mathrm{Ag}]\cdot[\mathrm{Ab}] \rightleftharpoons k_2\cdot[\mathrm{AgAb}]$$

where [Ag], [Ab] and [AgAb] represent antigen, antibody and complex concentrations respectively. The following assumptions were also made: (1) that the specific activity of the ^{131}I-labelled-antibody molecules was the same as that of ^{131}I-labelled γ-globulin; (2) that the molecular weight of the antibody was 160 000; (3) that anti-D was univalent;

(4) that the concentration of antibody-combining sites on the red cells was 450 $\mu\mu$moles/ml. of red cells (for the derivation of this value, see the Results section).

Suppression of non-specific uptake of ^{131}I *by red cells.* Hughes-Jones & Gardner (1962) showed that the non-specific uptake of ^{131}I (i.e. uptake of ^{131}I not due to ^{131}I-labelled antibody) was due partly to the non-specific adsorption of ^{131}I-labelled serum protein at the red-cell surface, partly to the exchange of ^{131}I-labelled lipid between the lipoproteins of the serum and the red-cell surface, and occasionally to the persistence of [^{131}I]iodide within well-washed red cells. A decrease of non-specific uptake was mainly achieved by the method suggested by Boursnell *et al.* (1953), namely competitive inhibition of uptake by the addition of at least a 15-fold excess of unlabelled serum proteins to the ^{131}I-labelled γ-globulin solutions. Further decreases of ^{131}I-labelled lipid and to some extent protein were also achieved by washing the cells at 0° eight times in approx. 10 vol. of a 1:10 dilution of human serum in 0·17 M-NaCl (Hughes-Jones & Gardner, 1962); the ^{131}I-labelled lipid on the red-cell surface exchanged with the lipid of the serum proteins. This method of removing ^{131}I-labelled lipid replaced the method described by Hughes-Jones *et al.* (1962), namely extraction with lipid solvents.

Purification of ^{131}I-*labelled antibody.* Purification was only achieved in the sense that ^{131}I-labelled antibody was freed from other ^{131}I-labelled compounds. This was carried out by dissociating ^{131}I-labelled antibody from red cells at pH 4·0.

As red cells are lysed at pH 4·0, it was convenient to lyse the cells initially by freezing at −79° and to wash the stroma four times at 2°. The stroma was then suspended in a 1% solution of serum proteins at 37°, the pH was adjusted to 4·0 and the suspension was left for 5 min. The stroma was then removed by centrifuging and the supernatant dialysed against 0·17 M-NaCl, pH 6·7.

In antibody solutions prepared in this way, between 90 and 99% of the ^{131}I was found to represent active antibody that would specifically combine with red cells. The uptake of ^{131}I by control rr cells was less than 2% of that taken up by the R_1R_1 cells under similar conditions.

Determination of the equilibrium concentration of antigen–antibody complex at various concentrations of free antibody. Purified ^{131}I-labelled antibody from serum Av was used for these experiments. Doubling dilutions of antibody were prepared and each dilution was added to 0·2 ml. of R_1R_1 and rr cells and incubated for 1 hr. at 37° with frequent shaking. The suspension was then spun and the supernatant removed, the cells were washed once with 1 ml. of 0·17 M-NaCl at 0°, and the ^{131}I contents of cells and supernatant were estimated. The percentage of ^{131}I representing active ^{131}I-labelled antibody in the purified antibody solutions was determined by repeatedly absorbing a sample of the antibody solution with R_1R_1 cells until no more ^{131}I was taken up by the cells (usually five absorptions). These reactions were carried out at pH 6·7.

Determination of the rate of dissociation of antibody from red cells. Formation of the antigen–antibody complex was first achieved by suspending 2 ml. of R_1R_1 red cells in solutions of ^{131}I-labelled antibody (non-purified). Dissociation of the complex was then carried out at 37° by suspending the cells in 3 ml. of 0·17 M-NaCl containing an excess of unlabelled antibody to inhibit the reassociation of ^{131}I-labelled antibody. At intervals the suspension was centrifuged, the supernatant removed and a fresh 3 ml. of unlabelled antibody solution added.

As a control for these experiments, 2 ml. of rr cells, which had also been suspended in the solutions of ^{131}I-labelled antibody, were treated in the same way. In the four experiments with serum Av the amounts of ^{131}I on the control rr cells compared with that on the R_1R_1 cells were 12, 2·5, 1·1 and 5% respectively.

The pH of the suspensions in these experiments lay between 7·3 and 7·5 at 37°.

Determination of the rate of association of antibody and antigen. The rate of association was measured by using purified preparations of ^{131}I-labelled antibody from serum Av. To obtain greater accuracy in the timing of the reaction, the rate was made as slow as possible by using low concentration of red cells and antibody, the limit of the dilution being determined by the ^{131}I concentration needed for accurate estimation of the radioactivity. Sufficient volumes of red cells and antibody solution (pH 6·7) were brought to the required temperature, rapidly added together and 3 ml. samples removed at intervals. The reaction was stopped by centrifuging at 1500 *g*. The precise moment of separating the cells from the free antibody could not be determined, but experiment had shown that it was approx. 20 sec. after starting the centrifuge. Correction for non-specific uptake of ^{131}I and for ^{131}I trapped in the red-cell mass was made from the results obtained with control rr cells.

RESULTS

Determination of the equilibrium concentrations of antigen–antibody complex at various concentrations of free antibody. Three experiments were carried out to determine the amount of antibody bound by the red cells at various concentrations of free antibody. In Fig. 1 the results have been plotted by the method of Scatchard (1949), according to the equation:

$$r/[A] = Kn - Kr$$

where r is the amount of antibody (moles/ml.) taken up by the red cells at equilibrium, [A] is the molar equilibrium concentration of free antibody,

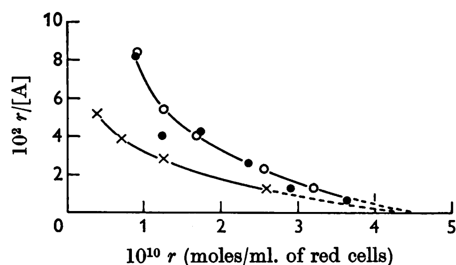


Fig. 1. Equilibrium relationships between the amount of antibody on the red cells, r (10^{-10} moles/ml.), and the ratio r/[A], where [A] is the molar concentration of free antibody. The results from three experiments are shown (●, ○ and ×) each with different preparations of antibody and three different sources of red cells. Experimental details are given in the text.

and n the number of moles of antibody combined with 1 ml. of cells when all the antigen sites are combined with antibody. If the curve is extrapolated to $r/[A] = 0$, then $r = n$, the maximum amount of antibody that can be taken up by the red cells. As the extrapolated part of the curve is long, only an approximate value can be given, namely 450 $\mu\mu$moles/ml. of red cells.

By assuming that each molecule of antibody only reacted with one antigen site and that the volume of a red cell is 90 μ^3, it was calculated that there were approx. 24 000 antigen sites on each red cell (probable genotype R_1R_1). The accuracy of this value is also limited by the assumption that the specific activity of the antibody is the same as that of γ-globulin. The plot of $r/[A]$ is curvilinear, indicating that there is heterogeneity of the intrinsic binding constant.

Determination of the rate of dissociation of antigen–antibody complex. Red cells were first combined with ^{131}I-labelled antibody; dissociation was then allowed to proceed at 37° in the presence of an excess of unlabelled antibody to prevent reassociation of the ^{131}I-labelled antibody. Four experiments were carried out with antibody from serum Av. All the results were similar and the results of one experiment are shown in Fig. 2. It is clear from the shape of the curve that there is considerable heterogeneity with respect to the rate constant for dissociation. Because of this heterogeneity, it is not possible to calculate exact values for the rate constants, but approximate values of the order and range of the rate constant, k_2, can be obtained from the minimal and maximal slope of the curves. Because of the heterogeneity, this method of calculation underestimates the total range of the rate constant. The minimal and maximal values of k_2 were calculated to be $1{\cdot}0 \times 10^{-5}$ and $1{\cdot}5 \times 10^{-4}$ sec.$^{-1}$ respectively at 37°.

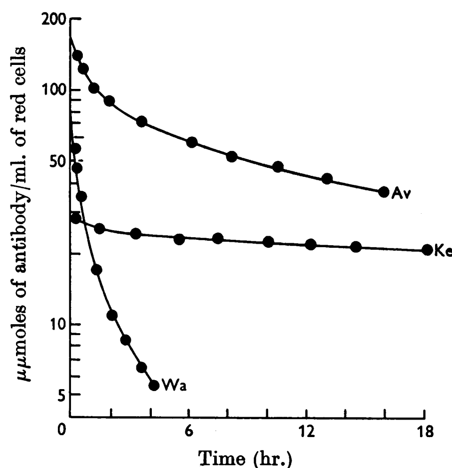


Fig. 2. Rate of dissociation of the antigen–antibody complex with antibody from serum Av, serum Ke and serum Wa. Experimental details are given in the text.

Similarly, observations were also made with antibody from sera Wa and Ke. These results are also shown in Fig. 2. It is clear that there were considerable differences in the rate of dissociation between these three sera. For antibody in serum Wa, the observed range of k_2 was from $3{\cdot}4 \times 10^{-4}$ to $6{\cdot}1 \times 10^{-5}$ sec.$^{-1}$; for the antibody in serum Ke the highest and the lowest values were $3{\cdot}6 \times 10^{-5}$ and $1{\cdot}1 \times 10^{-6}$ sec.$^{-1}$ respectively. The antibody of serum Ke dissociated so slowly at 37° that only 18% was removed during the 18 hr. incubation period, when the experiment was discontinued owing to the lysis of red cells. By studying the rate of dissociation of the complex with R_1R_1 cells from several different donors, it was found that the differences in the rate constants between the sera were due to differences between the antibodies and not the antigen.

Talmage (1960) suggested that, after the initial combination between an anti-albumin antibody and albumin, a recombination took place resulting in a firmer antigen–antibody bond. Three experiments were therefore carried out to obtain evidence of a second phase of association with the anti-D antibody. A sample of R_1R_1 cells was suspended in ^{131}I-labelled anti-D for 15 min. at 37°, washed and then divided into two parts, and the rate of dissociation of one part was carried out immediately. The other part was incubated at 37° for 6 hr. before determining the rate of dissociation.

In all three experiments it was found that, after incubation for 6 hr., the rate of dissociation during the first 2 hr. was slower by a factor of 2–3, but thereafter the rate of dissociation was the same as from those cells that had not been incubated. This indicated that at least part of the antibody had become more firmly bound.

Rate of association of antibody from serum Av. The rate of formation of antigen–antibody complex at 20° was determined with purified antibody from serum Av; the initial concentration of antibody was $0{\cdot}58$ μmM and that of antigen was $6{\cdot}6$ μmM; the result is shown in Fig. 3. Equilibrium was taken to have been obtained after 6 hr. The values for k_1, the rate constant for association, have been calculated for each observation by using the equation published by Hughes-Jones *et al.* (1962). The calculated values were found to fall progressively from $6{\cdot}3 \times 10^4$ l.mole^{-1} sec.$^{-1}$, calculated from the data obtained at 2 min., to $3{\cdot}0 \times 10^4$ l.mole^{-1} sec.$^{-1}$, from the data obtained at 160 min. The equation for calculating k_1 assumes that the rate constant for dissociation, k_2, is homogeneous. In fact k_2 has been shown to be heterogeneous. The effect of using a

single value of k_2 in the equation is to give an apparent heterogeneity of k_1. This effect is small, however, and cannot account for the apparent heterogeneity of k_1.

Comparison of the rates of association of antibody from sera Av, Ke and Wa. As only small amounts of the sera Ke and Wa were available, the rates of association were determined with the ^{131}I-labelled antibody that had been dissociated successively from red cells, as described above. Antibody from serum Av was used in the same way to obtain a direct comparison of the results. The presence of unlabelled anti-D antibody in these samples of dissociated ^{131}I-labelled anti-D was partially overcome by increasing the volume of red cells; nevertheless, the uptake of ^{131}I-labelled antibody was low and the ^{131}I content of the control cells relatively high (up to 20% of that of the R_1R_1 cells), which decreased the accuracy of the results. The concentration of antigen–antibody complex was plotted against time, and the rate constant was determined from the initial slope of the curve. Twelve successive samples of antibody from serum Av were investigated in this way; the average value of the rate constant for association, k_1, was $2{\cdot}1 \times 10^5$ l.mole^{-1} sec.$^{-1}$ at 37°. Similarly, the first eight samples dissociated from cells that had combined with antibody from serum Wa gave an average value of k_1 of $0{\cdot}9 \times 10^5$ l.mole^{-1} sec.$^{-1}$. So little antibody from serum Ke dissociated from the cells that it was only possible to estimate the approximate rate of association on the first two samples. Values of k_1 of $0{\cdot}5 \times 10^5$ and $1{\cdot}0 \times 10^5$ l.mole^{-1} sec.$^{-1}$ were obtained. Taking experimental error into account, it cannot be concluded that there is any significant difference in the initial rates of association of antibody obtained from the three different samples of antibody.

With these observed values of k_1 and k_2, it is possible to calculate the minimum range of the equilibrium constant, K, and the standard change in free energy, ΔF_0, for these sera. These values are given in Table 1.

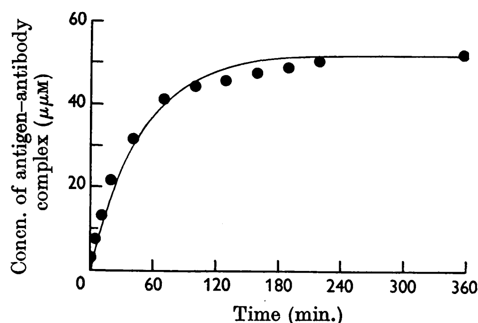


Fig. 3. Rate of formation of antigen–antibody complex at 20°. Experimental details are given in the text. The continuous curve is derived from the law of mass action, by using a single average value for the rate constant for association, k_1.

Table 1. *Estimated values of K and ΔF_0 at 37°, derived from independent measurements of k_1 and k_2*

	K (l.mole^{-1})	ΔF_0 (kcal./mole)
Serum Wa	$1{\cdot}4 \times 10^8$–$4{\cdot}5 \times 10^8$	$-11{\cdot}6$ to $-12{\cdot}3$
Serum Av	$0{\cdot}9 \times 10^9$–$14{\cdot}3 \times 10^9$	$-12{\cdot}7$ to $-14{\cdot}4$
Serum Ke	$0{\cdot}22 \times 10^{10}$–$7{\cdot}2 \times 10^{10}$	$-13{\cdot}3$ to $-15{\cdot}4$

Effect of temperature on the rate of association. The rate of formation of the antigen–antibody complex was determined at 2°, 10°, 20°, 31° and 40°. The pH of the reaction mixture was adjusted to 6·7 at each temperature. The concentration of antigen–antibody complex was plotted against time in each experiment and the rate of the reaction determined from the initial slope of the curve. The plot of ln k against $1/T$ according to the Arrhenius equation was linear. The energy of activation, E_A, was calculated to be 13 500 cal./degree/mole.

Effect of temperature on the equilibrium constant. The equilibrium constants were determined on four occasions with purified antibody of serum Av and R_1R_1 cells at 2°, 10°, 20°, 30° and 40°, and at pH 6·7. Because of the heterogeneity of the system, the equilibrium constants were only average values and were of the order of 1×10^9 l.mole^{-1} sec.$^{-1}$, which agrees well with the values calculated from the separate determinations of k_1 and k_2 when it is considered that the method of purification probably eliminates antibody with the highest binding constant. Over the range investigated, temperature had no significant effect on the value of K; the standard change in enthalpy, ΔH_0, was thus $0 \pm 0{\cdot}7$ kcal./mole. The standard change in entropy, ΔS_0, for antibody of serum Av was calculated to be 41 ± 2 cal./degree/mole.

DISCUSSION

By using Scatchard's derivation of the law of mass action, the average number of D antigen sites on each red cell of the probable genotype CDe/CDe was estimated to be approx. 24 000. This is greater than that found by previous workers; Boursnell *et al.* (1953) estimated the number to be 5500 and Masouredis (1960*b*) found 7400. These authors used ^{131}I-labelled antibody of relatively low titre and did not allow for the fact that all the antigen sites would not be bound by antibody at low concentrations of free antibody. The number of D antigen sites is considerably less than the number of c antigen sites, which have been estimated to be 65 000/red cell (Hughes-Jones *et al.* 1962).

There is much heterogeneity of the equilibrium constant, K, within a particular example of anti-

body, owing mainly to the heterogeneity of the rate constant for dissociation. For instance, there was only a twofold difference between the highest and lowest values of the rate constant for association of the antibody of serum Av, whereas the rate constant for dissociation shows a 15-fold difference between the highest and lowest observed values. Similarly, variations in the equilibrium constant between different examples of antibody can also be explained by the greater heterogeneity of the rate of dissociation of the complex. Thus, within experimental error, no significant difference was demonstrated between the initial rates of association in the three examples of antibody examined, whereas there was a tenfold difference between the initial observed rates of dissociation. This agrees with the findings on the antibody, anti-c (Hughes-Jones *et al.* 1962). The factors that may contribute to the heterogeneity of the rate constant for dissociation are: (1) heterogeneity of the antigen; (2) heterogeneity of the antibody; or (3) a variation in the strength of the antigen–antibody bond between reactants of similar structure as a result of the small variations in the spatial relationships between the complementary binding sites. Of these three reasons, it is likely that heterogeneity of the antibody plays a predominant role. Evidence for heterogeneity of the antibody depends on the separation of the antibody into fractions that have different rate constants for dissociation. This has been achieved for the blood-group antibody anti-c (Hughes-Jones *et al.* 1962), and it has been possible to obtain, by differential dissociation at acid pH, two fractions of anti-D antibody from serum Av which differ in their rate constants for dissociation (N. C. Hughes-Jones, B. Gardner & R. Telford, unpublished work). The finding that the bond strength increases on incubation at 37° suggests that there may also be some heterogeneity either of the structure of the antigen or of the bond strength between reactants with identical binding sites. The results of this experiment can best be explained, as was suggested by Talmage (1960) for the anti-albumin system, as being due to a more rapid dissociation of the weaker bonds during incubation followed by reassociation in which at least a portion of the antibody molecules makes a firmer bond, either by attachment to antigen whose structure is such that a stronger bond is formed, or by chance owing to a distribution of bond strengths between otherwise similar molecules.

Temperature was found to have no effect on the equilibrium constant of the antibody of serum Av. This is similar to the findings for the antibody anti-c (Hughes-Jones *et al.* 1962), and was also found for the antibody against the bivalent hapten terephthalanilide-*pp′*-diarsonic acid (Epstein, Doty & Boyd, 1956) and for an anti-(bovine albumin) antibody (Singer & Campbell, 1955). This indicates that there is a large increase in entropy of the system as the result of the formation of the complex, due either to the release of water of electrostriction or to rearrangement of atoms within the antibody molecule.

The rate of association of the antibody anti-D (approx. $2{\cdot}0 \times 10^5$ l.mole^{-1} sec.$^{-1}$ at 37°) is almost ten times as great as that found for the antibody anti-c ($3{\cdot}2 \times 10^4$ l.mole^{-1} sec.$^{-1}$ at 37°) (Hughes-Jones *et al.* 1962). Talmage (1960) found a value of approx. $2{\cdot}0 \times 10^4$ l.mole^{-1} sec.$^{-1}$ for an anti-albumin antibody (by assuming a valency of 6 for the antigen molecule). These values are lower than those found for the association between small haptens and antibody. For instance, Day, Sturtevant & Singer (1962) found a maximum value of 1×10^8 l.mole^{-1} sec.$^{-1}$ for antibody against the 2,4-dinitrophenyl group, and Froese, Sehon & Eigen (1962) a value of approx. 2×10^7 l.mole^{-1} sec.$^{-1}$ for antibody against phenylarsonic acid. The slower rate of association of the blood-group antibodies is probably in part a reflection of the fact that, in the hapten–antibody systems, the number of collisions between reactants will be mainly determined by the diffusion rate of the small hapten groups, which will be considerably greater than the rate of diffusion of the blood-group-antibody molecules towards the relatively static antigen attached to the red cells.

The energy of activation, 13 500 cal./degree/mole, is high for antigen–antibody reactions. The effect of temperature on the speed of reaction can also be expressed as the ratio, Q_{10}, of the speed of reaction at one temperature to that at a temperature 10° lower. In these terms, a value for Q_{10} of 2·5 is obtained for the temperature range 30–40°. This suggests that the rate-determining step in this antigen–antibody reaction is the actual combination of antigen and antibody to form the complex and not that of diffusion of the antibody towards the antigen. High values of Q_{10} have also been found for other antigen–antibody systems, namely, a Q_{10} of over 2·0 was found for an anti-virus antibody (Burnet, Keogh & Lush, 1937) and a value of 2·0 was found for an anti-bacteriophage antibody (Kalmanson, Hershey & Bronfenbrenner, 1942). On the other hand, Talmage (1960) found a value of 1·65 for an anti-albumin antibody, and Cann & Clark (1954) found a value of 1·4 (E_A = 6000 cal./degree/mole) for an anti-bacteriophage antibody. This latter value suggests that in this particular system the rate-limiting step is diffusion of the reactants towards each other, the effect of temperature on the rate of reaction being through its effect on the viscous flow of water. Although the higher values of Q_{10} obtained for bacteriophage-neutralizing sera have been criticized on technical grounds (Cann & Clark, 1954), it is possible that antigen–antibody systems differ as to their rate-limiting steps at 37°.

The maximal rate constant for association for the anti-D system can be calculated from the gas-collision formula of Moelwyn-Hughes (1947). A value of about 1×10^8 l.mole^{-1} sec.$^{-1}$ is obtained if it is assumed that: (1) the size of both combining sites is 700 Å^2, which is the upper limit given by Campbell & Bulman (1952) for the anti-(arsanilic acid) system; (2) that a collision occurs between combining sites if the lateral displacement between the centres of the combining sites is not more than twice the radius; (3) that every collision between combining sites results in union. This calculation does not take into account any steric factors concerned in the association of the reactants. The observed rate of reaction is thus slower by a factor of approx. 10^{-3} than the maximum theoretical rate.

SUMMARY

1. The reaction between three examples of the human blood-group antibody anti-D and red cells has been investigated by using ^{131}I-labelled antibody.

2. The range of the equilibrium constants, K, extended from $1 \cdot 4 \times 10^8$ to $7 \cdot 2 \times 10^{10}$ l.mole^{-1}.

3. The minimum range of the rate constant for association of one example of anti-D antibody was $3 \cdot 0 \times 10^4$–$6 \cdot 3 \times 10^4$ l.mole^{-1} sec.$^{-1}$.

4. The minimum observed ranges of the rate constant for dissociation for each of the three examples of anti-D were $3 \cdot 4 \times 10^{-4}$–$6 \cdot 1 \times 10^{-5}$, $1 \cdot 5 \times 10^{-4}$–$1 \cdot 0 \times 10^{-5}$ and $3 \cdot 6 \times 10^{-5}$–$1 \cdot 1 \times 10^{-6}$ sec.$^{-1}$.

5. The following thermodynamic constants were obtained for one example of antibody: standard enthalpy change, ΔH_0, $0 \pm 0 \cdot 7$ kcal./mole; standard entropy change, ΔS_0, 41 ± 2 cal./degree/mole.

6. The number of D antigen sites on each red cell of the probable genotype R_1R_1 was estimated to be approx. 24 000.

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The Effect of Calcium Ions on Ketone-Body Production by Rat-Liver Slices

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During a study of the factors responsible for the increased production of ketone bodies caused by starvation it was found that the presence of Ca^{2+} ions in the incubation medium profoundly affected the pattern of ketogenesis in liver slices. Calcium ions influenced the amounts of ketone bodies formed and the ratio of D(–)-β-hydroxybutyrate to acetoacetate, and the effect varied with the nutritional state of the animal and with the substrate metabolized.

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MATERIALS AND METHODS

Chemicals. Sodium pyruvate, prepared according to Robertson (1942), was dissolved in water immediately before use. Sodium *n*-octanoate was prepared by neutralization of redistilled octanoic acid. Acetoacetate, DL-β-hydroxybutyrate, enzymes and coenzymes were as described by Williamson, Mellanby & Krebs (1962).