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The Estimation of the Concentration and Equilibrium Constant of Anti-D

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Summary. A method is described for estimating the concentration and equilibrium constant of the blood group antibody, anti-D. The principle of the method is based on the fact that the reaction between anti-D and red cells is reversible and obeys the law of mass action. D-positive red cells at five different concentrations were added to aliquots of antisera containing anti-D and the reaction allowed to come into equilibrium. The amount of anti-D bound to the red cells in each case was estimated using ^{125}I-labelled anti-γ-globulin. The results of these estimates were then analysed according to two derivations of the law of mass action. Twenty-one examples of antisera containing anti-D were examined and the estimated range of values was as follows: concentration, 0·3–7·2 μg/ml; equilibrium constant, $1{\cdot}4 \times 10^7$ to $1{\cdot}2 \times 10^9$ l/mole; index of heterogeneity, a = 0·6–0·9.

INTRODUCTION

The reaction between anti-D and red cells is reversible and when equilibrium is reached antibody is present free in solution as well as bound to red cells. The extent of the agglutination produced by the addition of antiglobulin serum is dependent only on the amount of anti-D bound to the cells and an estimate of the titre of the antibody does not take into account the amount of antibody remaining free in solution. In any given mixture of D-positive red cells and anti-D, the amount of free antibody depends on the initial concentration of antibody, its equilibrium constant, and on the amount of red cells present. Similar considerations apply to the amount of anti-D which will combine with D-positive red cells in the circulation. Elucidation of the action of anti-D *in vivo* is thus dependent on a knowledge of the concentration and equilibrium constant in each particular case. This report presents a method of measuring these two factors.

The number of molecules of the Rh antibody, anti-D, bound to red cells can be estimated using iodinated anti-γ-globulin (Costea, Schwartz, Constantoulakis and Dameshek, 1962; Rochna and Hughes-Jones, 1965); this technique can be adapted to measure the anti-D content of serum. The principle of the method depends on the fact that the reaction between anti-D and red cells is reversible so that, at equilibrium, the amounts of bound and free anti-D are given by the law of mass action. Red cells at several different concentrations are added to aliquots of the serum containing anti-D and allowed to come into equilibrium. The amount of anti-D bound to the red cells in each case is estimated using ^{125}I-labelled anti-γ-globulin ([^{125}I]anti-γ-globulin) and the total antibody content of the

serum can be calculated by an analysis of the results according to a derivation of the law of mass action.

MATERIALS AND METHODS

Serum containing anti-D

Twenty-one sera, obtained from women at the time of delivery of a child suffering from haemolytic disease of the newborn, were kindly supplied by Dr W. Walker of Newcastle.

Red cells

Red cells (from a single CCDee donor) were used in all experiments for adsorption of the anti-D. The concentration of D antigen sites was estimated to be 313 p-moles/ml red cells by the method of Rochna and Hughes-Jones, 1965. The cells were washed three times before use, and white cells and platelets were removed.

[^{125}I]Anti-γ-globulin

Anti-human γ-globulin was obtained from a sheep and purified by absorption and elution from CM-cellulose to which human γ-globulin had been attached (Weliky, Weetall, Gilden and Campbell, 1964). This antibody was labelled with ^{125}I by the method of McFarlane (1958) and the combining ratio between this anti-γ-globulin and anti-D adsorbed onto red cells was estimated using an [^{131}I]anti-D. The anti-D used for calibration was obtained from a single donor. Details of these procedures and those used for determining specific activity and purity of the anti-γ-globulin are given elsewhere (Rochna and Hughes-Jones, 1965).

Procedure for estimating anti-D content of sera

The estimation of the anti-D content of sera is most accurate when there is approximately 0·2–2·0 μg of anti-D/ml of solution. It was found that antisera diluted 1:5 with 0·17 M NaCl, pH 7·0, usually gave a suitable concentration. If the initial estimate of antibody showed the concentration to be outside the optimal range, the procedure was repeated at the correct dilution.

Five 1-ml aliquots of the diluted antiserum were obtained and the R_1R_1 red cells were added in the following amounts, 2, 10, 50, 100 and 200 μl. The suspensions were then incubated at 37° for 2 hours with frequent mixing. This is sufficient time to bring the reactants into equilibrium. The suspensions were then centrifuged at 4° and the cells washed three times with 2 ml of ice-cold saline. One millilitre of [^{125}I]anti-γ-globulin (15 μg antibody/ml) was then added and after rapid mixing with a pipette, the suspensions were placed at 37° for 10 minutes without further disturbance. The suspensions were centrifuged and the supernatant saved for estimates of the free anti-γ-globulin concentration. The cells were transferred to another container with 2 ml of ice-cold saline, centrifuged, the supernatant removed and the ^{125}I content of the cells estimated. The amount of anti-D bound to the cells was then calculated using the previously determined combining-ratio between anti-D and anti-γ-globulin at the particular free anti-γ-globulin concentration present at the end of the 10-minute incubation period (see Rochna and Hughes-Jones, 1965).

Analysis of results

The results were analysed according to the derivation of the law of mass action derived by Scatchard (1949). The derivation is as follows: $r/[Ag]_{(free)} = Kn - Kr$ where r = the equilibrium concentration of antigen–antibody complex, n = the maximum amount of antigen–antibody complex that is formed when all the antibody is bound to antigen, K = the equilibrium constant and [Ag] = the free antigen concentration at equilibrium (derived as $[Ag_{(total)}] - [AgAb]_{(eq)} = [Ag]_{(free)}$). The value of n, which is equivalent to the total antibody concentration of the diluted antiserum, can be obtained by plotting $r/[Ag]$ against r and extrapolating to the abscissa, for, when $r/[Ag] = 0$, then $n = r$. In order to obtain the equilibrium constant, the data were analysed according to the derivation of Karush (1962) as follows: $\log r/n-r = a \log [Ag]_{(free)} + a \log K$. If $\log r/n-r$ is plotted against $\log [Ag]_{(free)}$, then the value of K is equal to $1/[Ag]_{(free)}$ when $\log r/n-r = 0$; the slope of the line gives the heterogeneity index, a. The molar concentration of antibody was calculated assuming a molecular weight of 160,000 for γG-globulin.

Antiglobulin titres on all twenty-one sera were estimated at the same time by a standard technique, using cells at a final concentration of 4 per cent and reading the results on a semi-opaque tile.

RESULTS

An example of the analysis of the results obtained in a typical case is shown in Figs. 1 and 2. The estimated concentrations, the values of the equilibrium constants and indices of heterogeneity are given in Table 1 and are shown graphically in Fig. 3. The relationship between titre and antibody concentration is shown in Fig. 4.

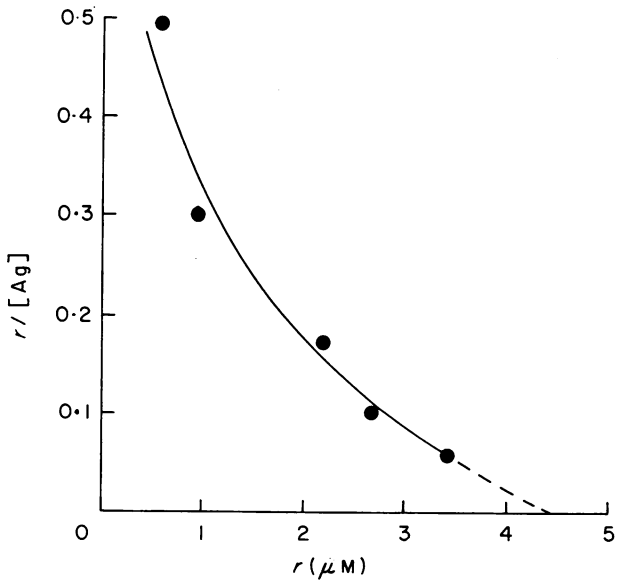

FIG. 1. An example of the plot of $r/[Ag]$ against r. The extrapolation to the abscissa gives the concentration of antibody in the diluted antiserum.

TABLE 1

THE ESTIMATED VALUES OF THE CONCENTRATION, EQUILIBRIUM CONSTANT (K) AND HETEROGENEITY INDEX (a) OF ANTI-D PRESENT IN TWENTY-ONE SERA OBTAINED FROM WOMEN AT THE TIME OF DELIVERY OF A CHILD SUFFERING FROM HAEMOLYTIC DISEASE OF THE NEWBORN

Serum	Concentration (μg/ml)	$K \times 10^{-7}$ l/mole	a
1	2·2	7	0·85
2	1·3	16	0·6
3	7·2	10	0·7
4	3·2	18	0·9
5	3·2	120	0·61
6	6·0	62	0·8
7	4·2	12	0·75
8	0·6	5	0·8
9	3·6	7	0·81
10	4·2	1·4	0·86
11	1·2	100	0·65
12	3·7	14	0·89
13	0·3	20	0·75
14	1·4	11·2	0·82
15	1·6	18	0·68
16	2·4	31	0·86
17	2·0	12	0·65
18	5·0	14	0·74
19	2·9	28	0·76
20	0·3	20	0·9
21	3·4	3·5	0·85

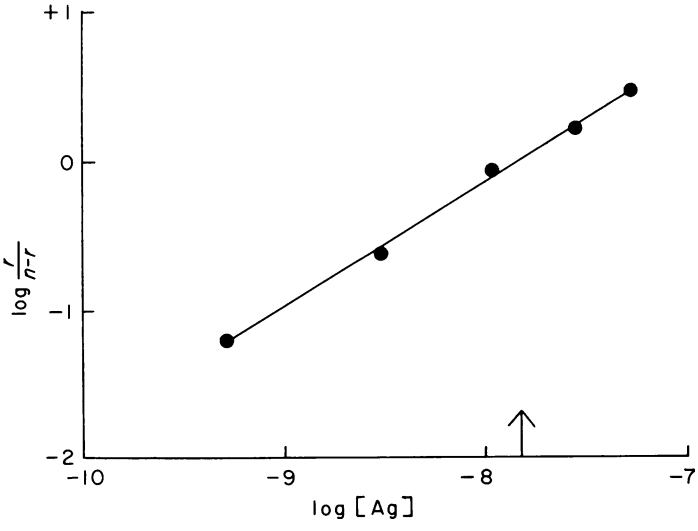

FIG. 2. An example of the plot of log $r/n-r$ against log[Ag]. The slope of the curve gives the heterogeneity index. The reciprocal of the molar concentration of [Ag] at the point when log $r/n-r = 0$ gives the value of the equilibrium constant K in l/mole.

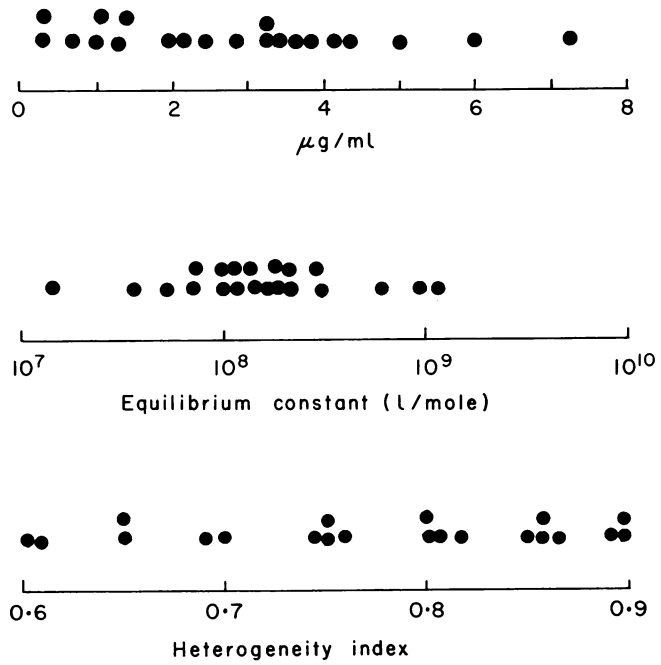

FIG. 3. Graphical representation of the estimates of antibody concentration, equilibrium constant and index of heterogeneity of twenty-one examples of anti-D.

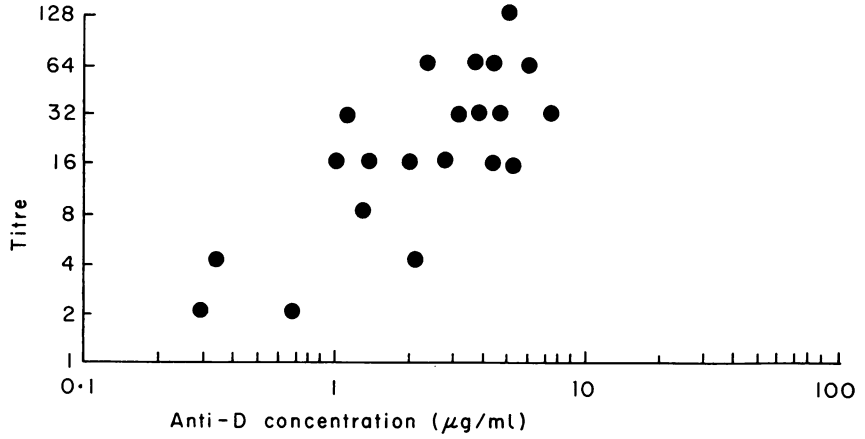

FIG. 4. The relationship between the anti-globulin titre and the anti-D concentration.

DISCUSSION

There are two main sources of error in this technique of measuring anti-D concentration. (1) The concentration must be estimated by extrapolation of a curved line, which could introduce an error of 10–20 per cent. (2) A more serious source of error derives from the assumption that the [^{125}I]anti-γ-globulin combined to the same extent with unknown examples of anti-D as it did with the [^{131}I]anti-D with which it was calibrated. An experiment was carried out to investigate the error introduced by this assumption. Ten

examples of anti-D were purified and labelled with ^{131}I and their combining ratio with the [^{125}I]anti-γ-globulin determined. It was found that there was a variation in the combining ratios of these ten examples compared with the original anti-D used for the calibration. Eight of the ten examples gave combining ratios within 25 per cent of that of the 'calibrating' anti-D, the other two gave ratios 40 per cent lower. The estimates of the anti-D concentration are thus probably within a factor of 0·6–1·5 of the true anti-D concentration.

Although the average values of the equilibrium constant vary widely, there being approximately a 100-fold difference between the lowest and highest values, twelve out of the twenty-one values fall within the narrow range $1{\cdot}0–3{\cdot}0 \times 10^8$ l/mole.

The heterogeneity index used here is that initially suggested by Nisonoff and Pressman (1958) and is based on the surmise that the distribution of equilibrium constants is normal or Gaussian around the mean. If this is so, then the plot of log $r/n-r$ against $\log[Ag]_{(free)}$ should be linear. Within experimental error, the data obtained in this investigation fit linear curves, and this provides evidence that the distribution does approximate to a normal curve. A heterogeneity index of $a = 1{\cdot}0$ indicates homogeneity, and values below indicate heterogeneity, the lower the value, the greater the heterogeneity. To give an indication of the range, it can be calculated (Fujio and Karush, 1966) that an antibody with a mean equilibrium constant of 1×10^8 l/mole and heterogeneity index $a = 0{\cdot}6$ will have two-thirds of all the molecules with constants within a range 1×10^7 to 1×10^9 l/mole and 95 per cent will be inside the range 1×10^6 to 1×10^{10} l/mole.

The relationship between titre and concentration of anti-D given in Fig. 4 shows that the correlation is poor, e.g. a titre of 32 being given by examples of anti-D in the range 1·2–7·2 μg/ml. There are two reasons for this poor correlation. (1) The error involved in the estimation of titre is recognized to be large; duplicate estimates frequently show a two-fold and sometimes a four-fold difference in strength. (2) The titre of an antibody not only depends on its concentration but also on the values of the equilibrium constant, the degree of heterogeneity and on the concentration of red cells. It has been found (unpublished observations) that at the end-point of the titration (standard anti-globulin test) there is approximately 1 μg anti-D/ml of red cells or 350 molecules/red cell. This is of the same order as that given by Dupuy, Elliot and Masouredis (1964). The titre gives no indication of the amount of antibody free in solution at the end-point, an amount which is dependent on the values of K and a. Thus, it can be calculated that two antibodies present at a concentration of 1 μg/ml but which have values of K equal to 1×10^7 and 1×10^9 l/mole would give titres of 2 and 22 respectively, provided that there was no heterogeneity of the equilibrium constant in each example. Antibodies are, however, always heterogeneous and if a value of $a = 0{\cdot}7$ is taken for each, then the titres become 4 and 20 respectively, provided that the final concentration of R_1R_1 cells used for the titre is 4 per cent. Thus equal concentrations of anti-D whose equilibrium constants fall at either end of the range found in this investigation would have a five-fold difference in titre.

The rate of red cell destruction *in vivo* caused by antibodies depends on the number of antibody molecules attached to red cells (Mollison and Hughes-Jones, 1967). The rate of destruction brought about by a particular antibody is thus dependent on its concentration and on the average value and the extent of heterogeneity of the equilibrium constant, that is, the same factors which affect the titre of an antibody. The titre and the biological activity of an antibody however are not necessarily related.

This results from the fact that the titre is usually estimated using a fixed quantity of red cells (4 per cent) whereas the number of red cells to be destroyed in any individual obviously varies from case to case. This can be illustrated as follows. Assuming that the minimal amount of anti-D on the red cells must be 0·03 μg/ml for destruction to take place (Mollison and Hughes-Jones, 1967) the total amount required in the plasma of an adult can be calculated for the two situations where there is either 1 ml or 400 ml of R_1R_1 cells in the circulation of an Rh negative individual. The values are shown in Table 2;

TABLE 2

TOTAL AMOUNT OF ANTIBODY (μg/3000 ml) WITH TWO DIFFERENT VALUES OF K WHICH ARE REQUIRED IN THE PLASMA TO GIVE A CONCENTRATION OF ANTI-D BOUND TO RED CELLS OF 0·03 μg/ml RED CELLS

D-positive red cells in circulation (ml)	Antibody	
	$K = 1 \times 10^7$ l/mole	$K = 1 \times 10^9$ l/mole
1	4·2 μg	0·2 μg
400	39 μg	13 μg

The heterogeneity index is taken to be $a = 0{\cdot}7$ in both cases.

it can be seen that with 1 ml of cells present in the circulation, 21 times as much of the weakly-binding antibody ($K = 1 \times 10^7$ l/mole) is required compared to the stronger binding antibody. When there are 400 ml of cells in the circulation only 3 times as much weakly-binding antibody is required as stronger binding antibody. These two antibodies give a five-fold difference in their titres. Titre and biological effectiveness would be related if the titre were estimated using the same concentration of D-positive cells as would be found in the circulation in any particular case *in vivo*.

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