RELATIONSHIP BETWEEN $Rh_0(D)$ GENOTYPE AND QUANTITY OF I^{131} ANTI-$Rh_0(D)$ BOUND TO RED CELLS *

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The antigens on the stroma of the red cell represent immunochemically specific molecules whose synthesis is genetically determined (1). The genetic control of the blood group antigens in man was recognized (2) within a decade after their discovery by Landsteiner. If the one-gene-one product hypothesis applies to the blood group antigens, the red cell antigen content should be related quantitatively to the genotype. The results obtained with techniques based on agglutination of red cells suggest that a dosage effect exists for many of the blood group systems (3). Some cells agglutinate more intensely than others with a given antibody indicating that these cells possess more antigen. Under these circumstances, it has been inferred that the red cells reacting more intensely with the antibody are genetically homozygous for the antigen whereas the weakly reacting cells are heterozygous.

Except for R^2R^2 (cDE/cDE) and $R^{ox}R^{ox}$ (-D-/-D-) red cells which react more strongly with anti-$Rh_0(D)$, no clear-cut dosage effect has been demonstrated for the $Rh_0(D)$ antigen (1). Grubb (4) using an antiglobulin absorption and inhibition technique was able to estimate the $Rh_0(D)$ receptor sites of the red cell but was unable to differentiate the heterozygous from the homozygous $Rh_0(D)$ red cell. Boursnell, Coombs and Rizk (5) have applied serum containing I^{131}-labeled anti-$Rh_0(D)$ to this problem. They did not derive any conclusions with respect to the $Rh_0(D)$ genotype because of the high nonspecific binding of I^{131}-labeled proteins to the red cells. Wiener and Gordon (6) have applied a quantitative antiglobulin test to this problem, but do not indicate that they were able to determine the $Rh_0(D)$ genotype with their technique.

The availability of a relatively specific I^{131} incomplete anti-$Rh_0(D)$ (7) provided the opportunity to study the relationship between the $Rh_0(D)$ genotype and the $Rh_0(D)$ antigen content of the red cell. The experimental design adopted for this study was to determine the $Rh_0(D)$ antigen content of different red cells obtained from the $Rh_0(D)$ positive donor population entering the Central Blood Bank of Pittsburgh. The frequency distribution of the red cell $Rh_0(D)$ content in this population of red cells was determined graphically, and the heterozygous state in this distribution was identified by studying genetically defined $Rh_0(D)$ heterozygotes obtained from family studies. This plan of study was used because there is no conventional technique [absence of both dosage effect and anti-rh (d) serum] for positively differentiating the $Rh_0(D)$ heterozygous red cell from the homozygous red cell. A preliminary report has been published (8, 9).

MATERIALS AND METHODS

Except for the procedures described below, the methods presented in a previous paper (7) were used for iodination,[1] determination of radioactivity, stroma preparation and reaction of the red cells with the I^{131} anti-$Rh_0(D)$.

Fractionation of anti-$Rh_0(D)$ serum. High titered anti-$Rh_0(D)$ serum was obtained from a 30 year old white housewife who had been immunized to the $Rh_0(D)$ antigen by multiple pregnancies. The pertinent details of her history and immunization have been presented (7).

The γ-globulins were isolated by the method of Nichol and Deutsch (10), in place of the modified Cohn method 10 procedure used previously (7). The titer of the anti-$Rh_0(D)$ serum before fractionation was 2,048 and the titer of the γ-globulin fraction was 2,048. The anti-

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[1] Carrier-free I^{131} was obtained from the Oak Ridge National Laboratory, Oak Ridge, Tenn.

TABLE I

Properties of the I^{131} anti-$Rh_0(D)$ γ-globulin fraction

Per cent of I^{131} bound	13.70
I (moles/mole globulin)*	0.98
I^{131} ($\mu c/\mu g$ N)†	0.15
Per cent of I^{131} precipitable with trichloroacetic acid	97.7
Titer before iodination	2,048
Titer after iodination	512
Nitrogen (mg/ml)	2.54

* Calculated using a molecular weight of 160,000 for the total fraction iodinated.

† Activity at the time of iodination.

$Rh_0(D)$ titers were determined with the antiglobulin reaction using pooled $Rh_0(D)$ positive red cells, volumetric dilutions of the anti-$Rh_0(D)$ preparation, and commercial antiglobulin reagent (11). The γ-globulin fraction contained 8.04 per cent of the total serum nitrogen as determined by the micro-Kjeldahl method (12). The properties of the I^{131} iso-antibody preparation are listed in Table I.

Elution of I^{131} anti-$Rh_0(D)$ from $Rh_0(D)$ stroma. A modification of the elution technique previously reported (7) was used for these studies. The pooled lyophilized $Rh_0(D)$ stroma was sensitized with the I^{131} anti-$Rh_0(D)$ γ-globulin at 37° C for 1 hour. To 10 g of stroma was added 100 ml of the I^{131}-globulin which contained 0.10 to 0.16 mg N per ml dissolved in pH 6.5, 0.15 M phosphate buffer with 0.03 per cent human γ-globulin carrier.[2]

The stroma, which had been sensitized in plastic tubes with a capacity of 30 ml was washed 4 times with the phosphate buffer by centrifugation (Spinco model L ultracentrifuge, 15 minutes at 20,000 rpm, maximum G 46,900).

The I^{131} anti-$Rh_0(D)$ was eluted from the sensitized stroma by adjusting the pH, temperature, and duration of elution. A preliminary eluate which contained mostly nonspecific I^{131}-globulins was obtained by eluting the sensitized stroma for 5 minutes at 56° C with pH 6.0, 0.15 M phosphate buffer containing 3 per cent γ-globulin carrier. This eluate was recovered by 15 minutes' centrifugation at room temperature (20,000 rpm, maximum G 46,900) and was discarded. The I^{131} anti-$Rh_0(D)$ was obtained by a second elution at 56° C for 30 minutes with pH 5.8, 0.15 M phosphate buffer containing 0.1 per cent γ-globulin carrier. The second eluate which contained biologically active I^{131} anti-$Rh_0(D)$ was separated from the stroma by centrifugation (15 minutes at 20,000 rpm, maximum G 46,900) in a Spinco model L ultracentrifuge using a rotor preheated to 60° C. The tubes containing the centrifuged stroma were placed in a 56° C water bath immediately after centrifugation and the supernates containing the eluted I^{131} anti-$Rh_0(D)$ were recovered by aspiration. The eluate obtained in this fashion was dialyzed overnight at 4° C against pH 6.5, 0.15 M phosphate buffer containing 1:10,000 merthiolate and 0.1 per cent γ-globulin carrier to readjust the pH of the eluates.

The eluates used in these studies were obtained from a pool of $Rh_0(D)$ positive red cell stroma derived from 16 units [3] of outdated whole blood. The Rh phenotypes of the units making up this pool were as follows: 7 units of R^1r (CDe/cde) or R^1R^0 (CDe/cDe); 4 units of R^2r (cDE/cde) or R^2R^0 (cDE/cDe); 3 units of R^1R^1 (CDe/CDe) or R^1r' (CDe/Cde) and 1 unit each of R^2R^2 (cDE/cDE) or R^2r'' (cDE/cdE), and R^2r (cDE/cde) or R^2R^0 (cDE/cDe). A total of 107 g of red cell stroma was obtained from these 16 units of whole blood.

Three different eluates were derived from this one pool of stroma. The properties of these eluates are listed in Table II. Eluate P-1 was obtained after reacting the globulin containing I^{131} anti-$Rh_0(D)$ (72 mg N) with 90 g of the stroma. Eluate P-2 was obtained by pooling 112 ml of P-1 and 40 ml of the supernates recovered from the studies in which $Rh_0(D)$ negative red cells had been reacted with P-1. Eluate P-3 was made up by pooling the $Rh_0(D)$ negative supernates derived from P-1 and P-2. This device of saving and pooling the supernates containing I^{131} anti-$Rh_0(D)$ from $Rh_0(D)$ negative red cells was used to achieve maximum utilization of the I^{131} anti-$Rh_0(D)$. The immunochemical activity of all three eluates was directly proportional to their total nitrogen content and was unaffected by previous reaction (absorption) with $Rh_0(D)$ negative red cells. The specific antibody content of each of the three eluates was determined by reacting the supernate with an excess of $Rh_0(D)$ positive red cells. The total antibody content was obtained by adding the antibody found in the supernate to that taken up by the original $Rh_0(D)$ positive red cell. These values are found in Table III.

The I^{131} γ-globulin fraction and the I^{131} anti-$Rh_0(D)$ eluate were studied by zone electrophoresis on potato starch with 0.1 ionic strength, pH 8.6 barbital buffer (13). Both the γ-globulin and the eluate preparations revealed a single symmetrical peak of radioactivity at the origin and a small albumin peak which had migrated away from the origin toward the anode. The distribution of protein in the starch block was associated with the I^{131} radio-

TABLE II

Properties of I^{131} anti-$Rh_0(D)$ eluates

Preparation	TCA-precipitable I^{131}*	Titer	Nitrogen
			μg/ml
P-1	87.0	16	3.96
P-2	86.1	16	3.83
P-3	89.2	4	2.92

* Trichloroacetic acid, 10 per cent.

[2] Obtained from Cutter Laboratories, Berkeley, Calif. The γ-globulin fraction was free of anti-$Rh_0(D)$ when tested with the antiglobulin reaction.

[3] These were conventional blood bank units consisting of 480 ml of whole blood and 120 ml acid citrate dextrose solution.

TABLE III

Antibody content of I^{131} anti-$Rh_0(D)$ eluates

Preparation	Nitrogen	No. of determinations	Antibody nitrogen*	Antibody nitrogen
	μg/ml		*μg/ml*	%
P-1	3.96	32†	0.127 ± 0.009	3.21
P-2	3.87	10	0.110 ± 0.015	2.85
P-3	2.92	4	0.0833 ± 0.0067	2.86

* Standard error of the mean.

† Seven of these determinations were based on reacting the supernates with the red cell suspension that was used for the original reaction. All the other values were obtained by reacting the supernates with a different $Rh_0(D)$ positive red cell sample.

activity. The antibody was localized on the cathode side of the protein peak at the origin.

Source of red blood cells. Red cells were obtained from the Caucasian donor population entering the Central Blood Bank of Pittsburgh and from the Blood Bank staff. The red cell samples were not accepted randomly from the donor population. $Rh_0(D)$ positive red cells were selected to conserve the supply of I^{131} anti-$Rh_0(D)$. Only 1 or 2 $Rh_0(D)$ negative samples were accepted each day to provide the necessary controls for the study. $Rh_0(D)$ negative cells or donors who did not know their Rh phenotype were rejected from the study. The red cell samples were obtained from venous blood anticoagulated with heparin. Some of the red cells were reacted with the I^{131} anti-$Rh_0(D)$ on the day they were collected and others were reacted after periods of 1 to 6 days of storage in Alsever's solution at 4° C. The cells were typed for the ABO and Rh antigens using commercial antisera [4] and the methods recommended by the manufacturer. The rh'(C) positive red cells included the $rh^w(C^w)$ antigen since a specific anti-$rh^w(C^w)$ was not used to test for this antigen.

TABLE IV

Experimental errors

Procedure	Degrees of freedom	Variance, 10^{-4} μg N/ 0.01 ml centrifuged RBC
Daily determinations on the same sample of stored red cells*	9	1.63
Daily determinations on the same unstored red cell sample*	5	2.28
Duplicate determinations after storage on different red cell samples	7	3.58
Duplicate determinations on the same cell suspension	11	0.678

* These data are derived from the results presented in Table II.

[4] I am grateful to the Ortho Research Foundation, Raritan, N. J. for generous supplies of anti-hr'(c).

A total of 354 individual determinations was carried out with the three eluates obtained from the pooled red cell stroma. The results to be discussed in this paper are based on the following studies: 199 different Caucasian red cell samples; 21 family study determinations; and 23 control determinations on $Rh_0(D)$ negative red cell samples. Eighty of the 354 determinations represented duplicate determinations, supernate studies and various miscellaneous studies. Excluded from this report are the studies carried out on 31 different samples of Negro red cells.

A series of five families with eight children in whom a documented history of Rh incompatible pregnancy was present was used for the source of $Rh_0(D)$ heterozygous cells.[5]

Calculations and presentation of results. The red cells were exposed to the I^{131} anti-$Rh_0(D)$ at 37° C for 1 hour. Cell concentration was determined by the microhematocrit method (14, 15). The results are expressed as micrograms of antibody nitrogen bound to 0.01 ml centrifuged red cells as defined by 5 minutes' centrifugation at maximum G 12,000 in an International micro-hematocrit centrifuge. The I^{131} bound to the red cells was converted to antibody nitrogen using the I^{131} to nitrogen ratio of the total γ-globulin fraction before elution. The nitrogen of this fraction could be determined with the micro-Kjeldahl procedure (12) but the nitrogen content of the eluates containing I^{131} anti-$Rh_0(D)$ was insufficient for direct determination. Justification for the conversion of I^{131} to nitrogen is based on the finding that the iodine to nitrogen ratio of specific antibody is the same as that of the total γ-globulin fraction (16). The I^{131} to nitrogen ratio has also been used to determine ultra-microgram quantities of I^{131} diphtheria toxin nitrogen (17). In this study the nitrogen values which were based on I^{131} radioactivity were confirmed independently by toxicity studies.

RESULTS

Effect of storage and over-all precision. The over-all reproducibility of the technique including the effects of storage was evaluated by storing a

[5] I am greatly indebted to Drs. Paul Gaffney and William Chapman of Pittsburgh, Pa., who made their patient material available for these studies.

large volume of red cells in Alsever's solution at 4° C. On subsequent days an aliquot of the stored cells was reconstituted and reacted with the I^{131} anti-$Rh_0(D)$. A fresh specimen of cells obtained by venipuncture from the donor of the stored cells was reacted along with the stored cells with the I^{131} anti-$Rh_0(D)$. The pooled variance for repeated determinations on the stored cells over a period of 11 days was 1.63×10^{-4} μg N per 0.01 ml RBC (Table IV) and the mean and standard error of the mean was $1.85 \pm 0.13 \times 10^{-2}$ μg N per 0.01 ml RBC [6] (Table V). The corresponding values for the determinations carried out on fresh unstored red cells were 2.28×10^{-4} (Table IV) and 1.89 ± 0.15 (Table V). There was no significant difference in the mean values between the fresh and stored red cell samples ($t = 0.18$, $n = 14$).

The over-all precision was evaluated in another way by repeating the determination on the same red cell suspension after a period of one to four days of storage at 4° C. The pooled variance for seven such different cells was 3.58×10^{-4} μg N per 0.01 ml RBC (Table IV).

The pooled variance for duplicate determinations on the same cell suspension for 11 different red cell samples was 0.678×10^{-4} μg N per 0.01 ml RBC (Table IV). This statistic excludes the errors that occur in preparing a cell suspension with a known hematocrit. It evaluates only the errors associated with the procedure of reacting the cells with the labeled iso-antibody—pipetting errors, loss of red cells both physically and by hemolysis during the procedure, and errors in the determination of radioactivity.

TABLE V

Effect of storage on quantity of antibody bound to the same sample of red cells

Age	Nitrogen bound, 10^{-2} μg/0.01 ml centrifuged RBC	
	Stored red cells	Fresh red cells
days		
0	1.79*	
1	1.75*	1.97*
2	1.85	1.79
3	1.97	1.89
4	2.08	
6	1.73	1.67
8	2.04	2.07
11	1.76	
Mean	1.85 ± 0.13	1.89 ± 0.15

* These values represent the average of duplicate determinations. The red cells used in this study were all obtained from one individual whose probable genotype was R^1r' (CDe/Cde).

[6] Mean and standard error of the mean are used throughout this paper.

TABLE VI

Number of red cells in 0.01 ml volume of centrifuged cells

Age	No. of samples counted	Mean number of cells $\times 10^7$ *
days		
0	5	9.37 ± 1.15†
1	15	8.44 ± 1.05
2	12	8.85 ± 1.93
3	8	9.07 ± 0.79
4	13	8.52 ± 1.63
5	8	9.10 ± 1.46
6	10	9.73 ± 2.04
7	4	8.75 ± 1.03
8	4	9.17 ± 1.14
9	6	10.13 ± 1.17

* The determinations were carried out with the Coulter electronic cell counter. The volume of centrifuged cells was defined by 5 minutes' centrifugation at maximum G 12,000.

† Standard error of the mean.

Relationship of hematocrit and cell count. The number of red cells in some of the cell suspensions was counted directly [7] by means of the Coulter electronic counter (18, 19). The cells had *in vitro* ages that ranged from 0 to 11 days. The mean number of red cells in a 0.01 ml volume of RBC, (defined by 5 minutes' centrifugation at maximum G 12,000) was $9.01 \pm 1.48 \times 10^7$ cells. The red cell count was directly proportional to the hematocrit even though there was considerable scatter in the data. The number of red cells was independent of the *in vitro* age of the red cell suspension (Table VI) indicating that the scatter in the data was not related to osmotic volume changes induced by the conditions of storage.

Nature and quantity of I^{131} bound to $Rh_0(D)$ negative red cells. The mean quantity of nitrogen bound to 23 samples of $Rh_0(D)$ negative red cells was $0.150 \pm 0.037 \times 10^{-2}$ μg nitrogen per 0.01 ml RBC. The quantity of nitrogen was independent of both the Rh or the ABO phenotype of both $Rh_0(D)$ and D^u negative cells, and represented from 3 to 7 per cent of the I^{131} bound to $Rh_0(D)$

[7] The interest and generous cooperation of Dr. R. C. Hamilton and of Sister Marietta (Registered Technologist, St. Francis Hospital, Pittsburgh, Pa.) in carrying out these determinations is gratefully acknowledged.

TABLE VII

Nitrogen bound to $Rh_0(D)$ negative red cells

Phenotype	No. of samples	10^{-2} μg Nitrogen bound to 0.01 ml centrifuged RBC
Distribution of samples by Rh phenotype		
rr (cde/cde)	16	0.160 ± 0.040*
r'r (Cde/cde)	3	0.130 ± 0.030
r''r (cdE/cde)	4	0.128 ± 0.015
Distribution of samples by ABO phenotype		
A	4	0.131 ± 0.034
B	3	0.126 ± 0.021
O	16	0.160 ± 0.038

* Standard error of the mean. The mean value for the 23 $Rh_0(D)$ negative red cells was $0.150 \pm 0.037 \times 10^{-2}$ μg N per 0.01 ml packed red cells.

positive cells. These results are tabulated in Table VII.

Criteria employed to determine antibody excess. The presence of antibody excess was ascertained by reacting $Rh_0(D)$ positive red cell supernates with unsensitized $Rh_0(D)$ positive red cells. The supernates were tested with either the same $Rh_0(D)$ positive cell or with an $Rh_0(D)$ positive cell obtained from a different individual (different Rh phenotype). All the tests on the supernates were carried out in the presence of a marked excess of antigen (large excess of red cells) so that an estimate of the total antibody content could be obtained.

Excess I^{131} anti-$Rh_0(D)$ could be demonstrated in all the supernates tested. The total antibody recovered by adding the antibody found in the supernate to that removed by the original $Rh_0(D)$ positive cell in six determinations was $0.126 \pm 0.016 \times 10^{-2}$ μg of antibody nitrogen per ml of I^{131} anti-$Rh_0(D)$. When the supernate was tested with a different $Rh_0(D)$ positive cell (26 determinations), the total antibody content was $0.127 \pm 0.008 \times 10^{-2}$ μg of antibody nitrogen per ml. These results indicate that the estimation of the total antibody content was independent of the type of $Rh_0(D)$ positive cell that was used to test the supernate. The value obtained using the same $Rh_0(D)$ positive red cell did not differ significantly from that found using a different positive red cell (Table VIII).

These data are plotted in Figure 1. The antibody nitrogen bound to the original red cell is plotted on the abscissa and the antibody nitrogen found in the supernate with the use of either the same cell or a different $Rh_0(D)$ positive red cell is shown on the ordinate. The quantity of antibody nitrogen remaining in the supernate was inversely proportional to the quantity of antibody removed by the original cell.

The degree of saturation of the available $Rh_0(D)$ sites on the red cell was also evaluated by re-reacting the washed I^{131} sensitized red cells with an additional quantity of I^{131} anti-$Rh_0(D)$. The increase in I^{131} anti-$Rh_0(D)$ bound to previously sensitized red cells after a second incubation of 60

TABLE VIII

Antibody excess as shown by free antibody in the supernate *

Phenotype original red cell	Antibody N bound to original red cell (A)	Phenotype red cell reacted with supernate	Antibody N found in supernate (B)		Total antibody N recovered (A+B)
R^1r or R^1R^0	4.40	Same cell	6.42		10.8
R^1r or R^1R^0	5.25	Same cell	5.88		11.1
R^1r or R^1R^0	8.84	Same cell	4.99		13.8
R^1r or R^1R^0	5.53	Same cell	6.04		11.6
R^1R^1 or R^1r'	8.59	Same cell	5.31		13.9
				Mean	12.2
R^1r or R^1R^0	5.34	R^1R or R^1r'	6.76		12.1
R^1r or R^1R^0	6.38	R^1R or R^1r'	5.80		12.2
R^1R^2 or $R'r''$	9.12	R^1R^1 or R^1r'	4.15		13.3
R^2r or R^2R^0	7.32	R^1R^1 or R^1r'	5.35		12.7
R^1R^1 or R^1r'	6.69	R^1R^1 or R^1r'	5.60		12.3
				Mean	12.5

* Antibody nitrogen in 10^{-2} μg N. The data have not been corrected for the nitrogen uptake of $Rh_0(D)$ negative red cells. Total nitrogen content of the I^{131} anti-$Rh_0(D)$ eluate was 3.96 μg N and 1 ml of a 1 per cent cell suspension was used for each reaction.

minutes was 10.33 ± 5.36 per cent (Table IX). This increase in antibody uptake following the twofold increase in reaction time is consistent with the kinetics of this reaction (*7*).

Frequency distribution of antibody nitrogen bound to $Rh_0(D)$ positive red cells. At the top of Figure 2 is a plot of the antibody nitrogen bound to 0.01 ml centrifuged red cells for a population of 47 $Rh_0(D)$ positive, rh'(C) negative red cells. On the basis of inspection the distribution can be divided into two groups by using the 3.2×10^{-2} μg N value. One group then consists of 37 different red cell samples with a mean value of $2.44 \pm 0.27 \times 10^{-2}$ μg N per 0.01 ml RBC, and the other group consists of 10 different red cell samples with a mean of 3.95 ± 0.34. The group of cells with the higher nitrogen values has an average nitrogen that is 1.62 times greater than that found in the group with the lower nitrogen values.

The two lower panels in Figure 2 present the distribution of two of the three common $Rh_0(D)$ positive, rh'(C) negative phenotypes. There are 14 red cell samples with an R^2R^2 or R^2r'' phenotype, 29 with an R^2r or R^2R^0 phenotype and 4 with an R^2r or R^0R^0 phenotype. There are insufficient numbers in each category to derive independent statistical values, but it is apparent that the distribution of antibody nitrogen values found in these different phenotypes does not differ from the distribution obtained by pooling all of the rh'(C) negative, $Rh_0(D)$ positive phenotypes.

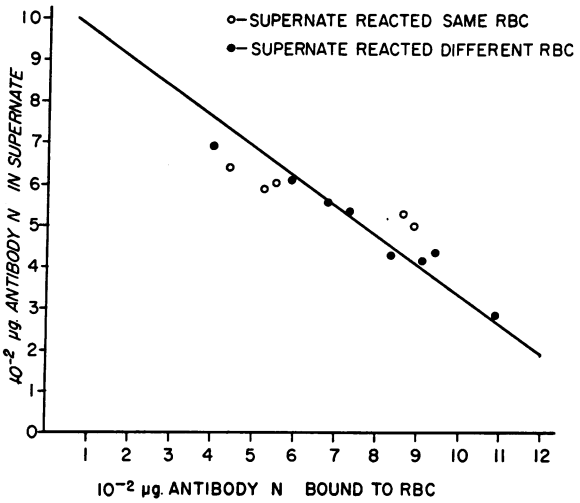

FIG. 1. RELATIONSHIP BETWEEN ANTIBODY N IN SUPERNATE AND ANTIBODY N BOUND TO RED CELLS. The antibody nitrogen bound to the $Rh_0(D)$ positive red cell is shown on the abscissa and the antibody nitrogen that was recovered in the supernate is plotted on the ordinate. The total quantity of antibody recovered in these supernate studies was unaffected by the Rh phenotype of the red cell that was used to test the supernate.

The frequency distribution of antibody nitrogen values in a population of 152 rh'(C) and $Rh_0(D)$ positive red cells is shown at the top of Figure 3. The bimodal distribution is evident but it is not as clearly defined as that found with the rh'(C) negative red cell. If 2.3×10^{-2} μg N is used to divide the two groups, one group has a mean value

TABLE IX

*Effect of reincubation on the quantity of antibody bound to red cells**

Probable red cell genotype	Nitrogen bound 10^{-2} μg N per 0.01 ml centrifuged RBC: After incubation for 60 min	After second incubation for 60 min	Per cent increase in nitrogen bound to red cells
R^1r or R^1R^0	1.76	1.80	2.3
R^1r or R^1R^0	2.10	2.28	8.6
R^1r or R^1R^0	3.54	3.79	7.1
R^1r or R^1R^0	2.17	2.34	7.8
R^1R^1 or R^1r'	3.31	3.59	8.5
R^1r or R^1R^0	1.53	1.88	22.9
R^1r or R^1R^0	2.55	2.87	12.6
R^1R^2 or R^1r''	3.65	4.13	13.2
R^2r or R^2R^0	2.87	3.18	10.1
R^1R^1 or R^1r'	2.68	2.96	10.5
		Mean	10.33 ± 5.36

* Red cells were washed and assayed for I^{131} after the first 60 minute incubation period and then were re-reacted with additional I^{131} anti-$Rh_0(D)$ for a second 60 minute period. One ml of the I^{131} anti-$Rh_0(D)$ with a total nitrogen content of 3.96 μg was used in both reactions and 0.5 ml of a 5 volumes per cent cell suspension was employed. The data have not been corrected for the nitrogen uptake of $Rh_0(D)$ negative red cells.

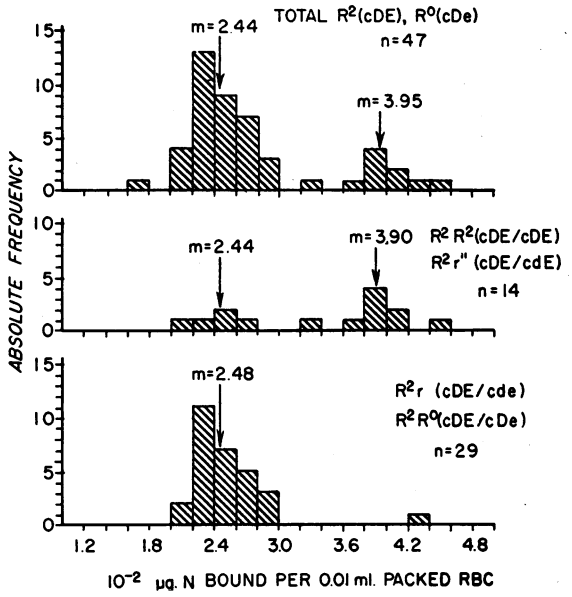

FIG. 2. DISTRIBUTION OF ANTIBODY N BOUND TO $Rh_0(D)$ POSITIVE, RH'(C) NEGATIVE RED CELLS. The bar graph at the top of this figure presents the findings for the total group of rh'(C) negative red cells. The two lower bar graphs are the results plotted separately for two of the common phenotypes.

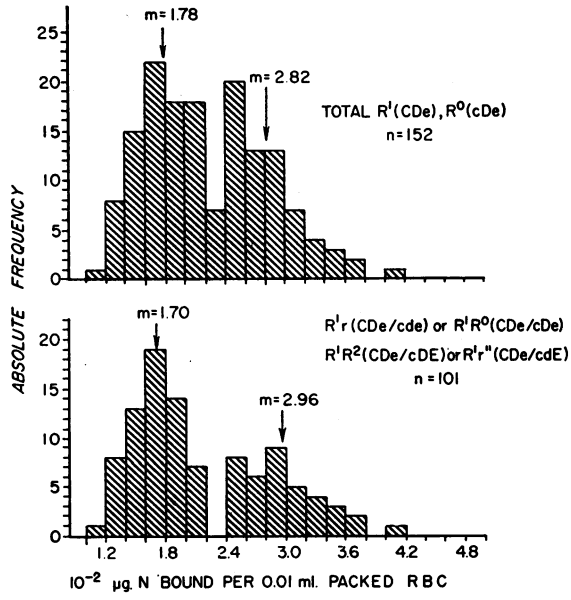

FIG. 3. DISTRIBUTION OF ANTIBODY N BOUND TO $Rh_0(D)$ AND RH'(C) POSITIVE RED CELLS. The bar graph at the top of the figure presents the findings for the total group of $Rh_0(D)$ and rh'(C) positive red cells. The lower plot presents the data obtained on only the heterozygous rh'(C) red cell samples.

of $1.78 \pm 0.03 \times 10^{-2}$ µg N per 0.01 ml RBC and the other group 2.32 ± 0.05. These values are significantly less than the corresponding values for the rh'(C) negative red cells, 2.44 and 3.95. The effect of the homozygous rh'(C) positive red cells on this distribution is shown at the bottom of Figure 3. The homozygous rh'(C) positive red cells have been excluded from this graph so that only the values for the heterozygous rh'(C) positive red cells are shown. A total of 101 different

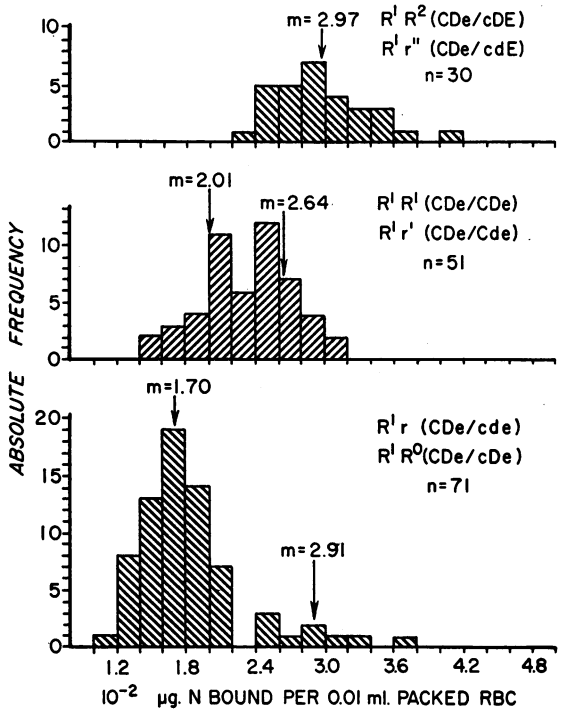

FIG. 4. DISTRIBUTION OF ANTIBODY N BOUND TO $Rh_0(D)$ AND RH'(C) POSITIVE RED CELLS. The results obtained for each of the three common $Rh_0(D)$ and rh'(C) positive phenotypes are shown in each bar graph. The data used to plot the bar graph at the top of Figure 3 were obtained by combining these three groups.

red cell samples has been plotted of which 71 had an R^1r or R^1R^0 phenotype and 30 had an R^1R^2 or R^1r' phenotype. In this case 62 of the 101 cell samples have a mean value of 1.70 ± 0.03 and 39 have a mean value of 2.96 ± 0.07, 10^{-2} µg N per 0.01 ml RBC.

The distortion in the bimodal distribution introduced by the rh'(C) red cell is shown by the data plotted in Figure 4. In this plot the three common rh'(C) positive red cell phenotypes are shown individually. At the top of the figure are 30 R^1R^2

TABLE X

Chromosome frequencies

Chromosome	Calculated from total sample* (n = 275)	Calculated from cells studied† (n = 210)	Reported by Race and Sanger (1)
r (cde)	0.3133	0.2070	0.3886
r′ (Cde)	0.0114	0.0012	0.0098
r″ (cdE)	0.0170	0.0012	0.0119
R^0 (cDe)	0.0224	0.0418	0.0257
R^1 (CDe)	0.4596	0.4873	0.4076
R^z (CDE)	0.0038	0.0005	0.0024
R^2 (cDE)	0.1725	0.2611	0.1411

* Includes the red cell samples from the family studies and some samples that had been phenotyped but which had not been reacted with the I^{131} anti-$Rh_0(D)$.

† Excludes the family study samples and includes only the red cell samples that were reacted with the I^{131} anti-$Rh_0(D)$.

or R^1r'' phenotypes; the middle plot shows 51 R^1R^1, or R^1r' phenotypes; and the graph at the bottom presents the findings on 71 R^1r or R^1R^0 red cell samples. The homozygous rh′(C) positive red cells (middle graph, Figure 4) show the poorest separation into two groups. If this distribution is divided arbitrarily into two groups by the 2.3×10^{-2} μg N value the standard error of the mean for each group can be determined and the two groups can be compared by the *t* test. Under these conditions the two groups are significantly different, $p < 0.05$. The heterozygous rh′(C) positive red cells show a better separation into two groups, one group with a mean nitrogen value of 1.70 and the other group with a mean nitrogen value of 2.91.

Chromosome frequencies. The chromosome frequencies for these data were calculated using the method described by Race and Sanger (1). Unfortunately, valid chromosome frequencies cannot be derived from these results since the calculations are not based on a random sample of Caucasian red cells. The red cells used for these studies were derived from a special population (blood donors), only $Rh_0(D)$ positive red cells were selected and during the course of the study a conscious effort was made to exclude the rh′(C) positive red cells. The effect of these selection criteria on the chromosome frequencies is shown in Table X. If all the red cell samples, including the family studies, are used for the calculations the chromosome frequencies are close to those reported by Race and Sanger (1). This agreement, however, is spurious because the exclusion of the $Rh_0(D)$ negative red cells from the data was compensated for by the family studies which contained an excess of $Rh_0(D)$ negative cells. The bias introduced by the devices used to select the red cells for this study is evident when the chromosome frequencies are derived from only the red cells that were reacted with the I^{131} anti-$Rh_0(D)$ and from which the family studies had been excluded. These values found

TABLE XI

Family studies

Father		Mother		Children	
Phenotype	N bound*	Phenotype	N bound*	Genotype	N bound*
R^1r'' (CDe/cdE)		rr (cde/cde)	0.17	R^1r (CDe/cde)	2.22
R^1R^z (CDe/cDE)	3.41			R^1r (CDe/cde)	2.48
R^zR^z (cDE/cDE)		r″r (cdE/cde)	0.15	R^zr'' (cDE/cdE)	1.86
R^zr'' (cDE/cdE)	2.96			R^zr'' (cDE/cdE)	1.50
R^1r (CDe/cde)	1.47	r″r (cdE/cde)	0.13	rr(cde/cde)	0.26
R^1R^0 (CDe/cDe)					
R^1r' (CDe/Cde)	†	r′r (Cde/cde)	0.10	R^1r' (CDe/Cde)	1.49
R^1r (CDe/cde)				r′r (Cde/cde)	0.14
R^1r (CDe/cde)	1.81	R^1r (CDe/cde)	1.90	rr (cde/cde)	0.16
R^1R^0 (CDe/cDe)		R^1R^0 (CDe/cDe)			

* 10^{-2} μg N per 0.01 ml centrifuged red cells.

† Father not available for study.

TABLE XII

Nonreacting $Rh_0(D)$ positive red cells

Phenotype	10^{-2} μg N bound to 0.01 ml red cells*
O, r″r (cdE/cde), D^u pos.	0.17
O, r′r (Cde/cde), D^u pos.	0.24
B, R^0r (cDe/cde) or R^0R^0 (cDe/cDe)	0.53
O, R^0r (cDe/cde) or R^0R^0 (cDe/cDe)	0.32

* The mean nitrogen bound to $Rh_0(D)$ negative red cells has not been subtracted from the values in the table.

in the second column of Table X differ considerably from the values expected for a Caucasian population (last column, Table X).

Family studies. The data obtained by studying the red cells of five families with eight children are shown in Table XI. The first four families involve $Rh_0(D)$ incompatible mating in which the father is $Rh_0(D)$ positive and the mother is $Rh_0(D)$ negative. Of the seven children that were studied, two were $Rh_0(D)$ negative and five were $Rh_0(D)$ positive. Four of the five heterozygous $Rh_0(D)$ positive red cell samples obtained from these children took up a quantity of antibody nitrogen that was within the lower peak of the bimodal distribution found in the population study. The R^1r red cells of one child had a value, 2.48 $\times 10^{-2}$ μg N, that was slightly greater than the 2.3×10^{-2} value which was used to divide the distribution of this particular phenotype into two groups.

In one family both the mother and child were $Rh_0(D)$ negative and the father was $Rh_0(D)$ positive. The antibody nitrogen bound to the father's heterozygous $Rh_0(D)$ red cells was within the lower nitrogen value group. In the last family shown on Table IV the child was $Rh_0(D)$ negative and the parents were $Rh_0(D)$ positive. The heterozygous $Rh_0(D)$ positive red cells of both parents had nitrogen values that were within the lower nitrogen peak of the bimodal distribution.

Reactivity of the red cell used to immunize. Two different red cells involved in the immunization of the antiserum donor were available for testing with the I^{131} anti-$Rh_0(D)$. One of the cells was the husband's red cell whose phenotype was A, R^1R^1 (CDe/CDe) or R^1r' (CDe/Cde). The other cell, A_1, R^0r (cDe/cde) or R^0R^0 (cDe /cDe), was the red cell used to reimmunize the donor. Both of these cells were reacted with the I^{131} anti-$Rh_0(D)$ to see if their reactivity was consistent with that found with other $Rh_0(D)$ positive red cells of the same Rh phenotype. The husband's cells took up 2.70×10^{-2} μg N per 0.01 ml RBC and the red cells used as the "booster" antigen took up 2.26. Both of these values fell within the range of values obtained with other red cells of similar Rh phenotype.

$Rh_0(D)$ variants. Of the 278 different $Rh_0(D)$ positive red cells studied, four different cell samples were found that did not take up significant quantities of the I^{131} anti-$Rh_0(D)$. Two of these cell samples were D^u positive and two were D^u negative. The phenotypes and quantity of anti-

TABLE XIII

*Assignment of antibody nitrogen values**

Phenotype	Heterozygous $Rh_0(D)$		Homozygous $Rh_0(D)$	
	Nitrogen†	No.‡	Nitrogen†	No.‡
R^2R^2 or R^2r''	2.44 ± 0.08	5	3.90 ± 0.11	9
R^2r, R^2R^0, or R^0r''	2.48 ± 0.05	28	4.38	1
R^0r or R^0R^0	2.18	4		
Total R^2, R^0	2.44 ± 0.04	37	3.95 ± 0.11	10
R^1r, R^1R^0, or R^0r'	1.70 ± 0.03	62	2.91 ± 0.16	9
R^1R^1 or R^1r'	2.02 ± 0.04	23	2.64 ± 0.04	28
R^1R^2, or R^1r'' or R^2r'		0	2.97 ± 0.08	30
Total R^1, R^2	1.78 ± 0.03	85	2.82 ± 0.05	67

* The table is based on red cells derived from 199 Caucasian individuals.
† The nitrogen value represents the mean and standard error of the mean in units of 10^{-2} μg N bound to 0.01 ml RBC.
‡ Represents the number of different red cell samples of the phenotype indicated which were used to determine the mean nitrogen value.

body nitrogen taken up by these four red cell samples are shown in Table XII. These four samples took up only 10 to 15 per cent of the nitrogen that was bound to the other $Rh_0(D)$ positive red cell samples, and in all cases, except one, these cells took up more I^{131} than did the $Rh_0(D)$ negative red cells. All four cell samples were negative by the indirect antiglobulin test after reaction with the I^{131} anti-$Rh_0(D)$. The original serum and the I^{131}-γ-globulin fraction before the absorption-elution procedure, however, gave positive indirect antiglobulin reactions with these cells. Although an antibody to the $Rh_0(D)$ antigen present in these red cells was available in the original antiserum, it apparently was lost during the stroma absorption and elution procedure.

Assignment of genotype to antibody values. Table XIII presents a summary of the $Rh_0(D)$ red cell antigen content of the common $Rh_0(D)$ genotypes. These values were derived from the study of 199 samples of Caucasian red blood cells and the genotype was assigned to these values using the data obtained from the family studies.

DISCUSSION

The $Rh_0(D)$ antigen content of the red cell has been estimated from the quantity of I^{131} incomplete anti-$Rh_0(D)$ bound to the red cell. A bimodal distribution of the red cell $Rh_0(D)$ content was found in a population of 199 different $Rh_0(D)$ positive red cell samples. Family studies indicate that the two groups of red cells are related to the $Rh_0(D)$ genotype of the individual from whom the cells were obtained. The segregation into two distinct populations was most evident for the red cells free of the rh'(C) antigen. The rh'(C) positive $Rh_0(D)$ containing red cells differed from the rh'(C) negative cells in two ways. There was an absolute decrease in total $Rh_0(D)$ antigen content as defined by the quantity of I^{131} anti-$Rh_0(D)$ bound to these cells and the cells were not as sharply separated into two groups. The homozygous rh'(C) red cells showed the poorest separation into two groups (middle bar graph in Figure 4). The $Rh_0(D)$ homozygous red cell in this case had a greater decrease in $Rh_0(D)$ content than did the heterozygous $Rh_0(D)$ cell. The nonproportional decrease of the $Rh_0(D)$ antigen in the two genotypes resulted in overlapping of the two distributions, so that a separation of the two $Rh_0(D)$ genotypes was not as clearly defined as that observed in the other Rh genotypes.

The suppressive or inhibiting effect of rh'(C) on the quantitative expression of the $Rh_0(D)$ property has been recognized by many investigators (20–22). This study is inadequate to explain the effect of the rh'(C) antigen on the quantity of I^{131} anti-$Rh_0(D)$ bound to rh'(C) positive red cells. The decrease in antibody uptake by these cells may be due to an immunological effect exerted by the rh'(C) antigen or the effect may be due to a genetic mechanism. Current genetic theories postulate that the rh'(C) effect represents a decreased elaboration of the $Rh_0(D)$ antigen resulting from either competition for a common substrate (22) or from some interaction between alleles at the Rh locus (21). The effect of rh''(E) on the quantity of I^{131} anti-$Rh_0(D)$ bound to rh''(E) positive red cells cannot be evaluated adequately since only four samples of R^0r (cDe/cde) or R^0R^0 (cDe/cDe) red cells were studied. The precise analysis of the interaction of the $Rh_0(D)$, rh'(C) and rh''(E) antigens must await more detailed family studies and a technique for the direct determination of the $Rh_0(D)$ red cell antigen content.

An effort was made to critically evaluate and experimentally control the two obvious limitations associated with the use of I^{131} anti-$Rh_0(D)$ to estimate the red cell $Rh_0(D)$ antigen content. One is the experimental error resulting from the techniques used to handle particulate suspensions (cells) quantitatively and the other is the immunochemical variability or heterogeneity of the $Rh_0(D)$ antigen.

The errors introduced into the results consist of those due to the experimental procedures and those that may result from the use of the hematocrit as an index of the number of cells present in the cell suspension. The assumption that the hematocrit is directly proportional to the number of cells implies that there is no change in cell volume during the study and that there is uniformity of red cell volume from one individual to the next. The results obtained in the storage experiments (Table V) indicate that there was no demonstrable change in cell volume during storage and that the reactivity of the antigen sites was unaffected by

either the conditions of storage or the procedures used in these studies.

The errors due to the experimental procedures can be evaluated from the variances listed in Table IV. These results indicate that less than one-fifth of the total variance is due to the procedure in which the red cells are reacted with the I^{131} anti-$Rh_0(D)$. The major source of error is associated with the determination of the hematocrit of the red cell suspension that is used in the reaction with the labeled antibody.

A reliable estimate of the number of antigen sites which is based on the quantity of cell-bound antibody requires that the reaction be in antibody excess. Insufficient antibody to saturate all the available antigen sites in the red cell suspension would lead to an underestimation of the antigen sites. The presence of antibody excess in these experiments was determined in two ways; in one case, the supernates from $Rh_0(D)$ positive cells were tested for free antibody, and in the other method, the sensitized, presumably saturated red cells, were re-reacted with additional antibody. The results obtained by both techniques showed that excess antibody was present (Tables VIII and IX).

The quantity of I^{131} taken up by $Rh_0(D)$ negative red cells is one of the possible indices of the specificity of the I^{131} bound to $Rh_0(D)$ positive red cells. The amount of I^{131} taken up by the $Rh_0(D)$ negative red cells was less than 6 per cent of the I^{131} bound to $Rh_0(D)$ positive red cells and was independent of the Rh or ABO phenotype (Table VII). Although previous absorption of the I^{131} anti-$Rh_0(D)$ negative red cells does not affect the content of anti-$Rh_0(D)$, the nature of the I^{131} protein bound to the $Rh_0(D)$ negative red cells has not been defined by these experiments.

The specificity of the anti-$Rh_0(D)$ was also evident in the experiments carried out on the $Rh_0(D)$ positive red cell supernates. A constant quantity of antibody was recovered when the antibody found in the supernate was added to the antibody taken up by the original red cell. This was true irrespective of the type of $Rh_0(D)$ positive red cell used to absorb the antibody remaining in the supernate (Table VIII and Figure 1). The total recovery of antibody was, in addition, independent of the amount removed by the first $Rh_0(D)$ positive red cell. These findings indicate that the $Rh_0(D)$ quality defined by the I^{131} anti-$Rh_0(D)$ was specific and consistent with a single anti-$Rh_0(D)$ which was absorbed stoichiometrically by a large number of $Rh_0(D)$ positive red cells.

The heterogeneity of the $Rh_0(D)$ antigen has been documented by many investigators (1). Four $Rh_0(D)$ positive red cell samples that did not react with the I^{131} anti-$Rh_0(D)$ were found in this series. The occurrence of these cells indicates that the antibody preparation used in this study was selected for a given quality or qualities of the $Rh_0(D)$ antigen by the techniques employed to obtain the antibody. Two independent operations served to define the $Rh_0(D)$ specificity of the I^{131} anti-$Rh_0(D)$. Specificity was determined by the nature of the antigens that were used to immunize the donor (multiple pregnancies and intentional re-immunization) and by the $Rh_0(D)$ antigen content of the red cell stroma used to concentrate the I^{131} anti-$Rh_0(D)$. The use of a pool of red cell stroma derived from many $Rh_0(D)$ positive red cell samples was an attempt to minimize the selective role of the stroma absorption and elution technique. In spite of these precautions it is evident that the I^{131} anti-$Rh_0(D)$ used in this study did not contain antibodies to all the $Rh_0(D)$ antigens.

Additional evidence for the heterogeneity of the $Rh_0(D)$ antigen was the distribution of the $Rh_0(D)$ antigen content in Negro red cells. The $Rh_0(D)$ antigen content in these red cells showed a distribution different from that found in Caucasian red cells. Inadequate numbers of such cells have been studied to determine if the difference noted is significant. Such a difference is consistent with the recognized difference in the frequency of many of the blood group antigens in the Negro (23).

The gene-product determined by these studies (blood group antigen) is restricted to the configuration of the antibody combining site on a mucopolysaccharide molecule if the $Rh_0(D)$ antigen resembles the ABO antigens (24, 25). Although a high degree of specificity exists for this site, it probably represents only a very small fraction of the total molecule (26, 27). As a result, only a fragment of the gene-controlled molecule is visualized so that no inference concerning the rest of the molecule can be drawn from these studies. It is conceivable that the antigenic configurations responsible for the different blood

group specificities are all found on a common molecule. The concept of such a basic blood group nucleus has been postulated for the $Rh_0(D)$ antigens (28). If experimental support can be obtained for this concept then the basic blood group nucleus would represent a molecule whose synthesis is dependent on many genes. The blood group antigens would then resemble other gene-controlled molecules, such as hemoglobin, which are believed to be under the control of several different genes (29). This resemblance, however, may be superficial in that the hemoglobin loci control primary protein structure (30), whereas the immunochemical specificity of the blood group substances could be due to changes confined to only the secondary protein structure.

An estimate of the number of antigen molecules or sites can be derived from these data if certain assumptions are granted. If the specific antibody iodinates to the same extent as the total γ-globulin fraction (16) and if 1.6×10^5 is used for the molecular weight of the $Rh_0(D)$ iso-antibody, the homozygous $Rh_0(D)$ cell contains about 10,300 antigen sites and the heterozygous cell about 6,400 antigen sites. For the rh′ (C) positive cell these values are 7,400 and 4,600, respectively. These values, although larger, are of the same order of magnitude as those that have been estimated with the techniques employed in previous studies (4–7). It will be of interest to determine the quantity of antibody bound to an individual cell to check the validity of these values which are based on averages.

The results obtained with the application of these techniques to the $Rh_0(D)$ antigen indicates that the use of an I^{131} labeled iso-antibody offers an exquisitely sensitive method for studying the distribution, quantity and specificity of the blood group antigens. The ability to quantitatively evaluate these parameters provides a means of investigating the genetic and immunochemical interrelationships of the substances of the blood groups. An obvious clinical application of this study has been made to the problem of hemolytic disease of the newborn due to Rh incompatibility. The genotype of $Rh_0(D)$ positive fathers has been determined using the data of this study so that a prognosis could be made with regard to future pregnancies.

CONCLUSIONS

1. An estimate of the $Rh_0(D)$ antigen content of 199 different Caucasian red cells was obtained from the quantity of I^{131} trace labeled incomplete anti-$Rh_0(D)$ bound to the red cells.

2. The validity of the technique was evaluated by defining the experimental errors, the $Rh_0(D)$ specificity of the I^{131} anti-$Rh_0(D)$, and the quantitative relationship between volume of red cells, quantity of I^{131} anti-$Rh_0(D)$ bound to the red cells, and the I^{131} anti-$Rh_0(D)$ that could be demonstrated in the supernate.

3. The quantity of I^{131} anti-$Rh_0(D)$ bound to rh′(C) negative, $Rh_0(D)$ positive red cells showed a bimodal distribution. The group of cells with the higher antigen content (1.6 times) was identified with the homozygous state [$Rh_0(D)$, $Rh_0(D)$] and the other group with the heterozygous state [$Rh_0(D)$], rh(d), by using genetically defined heterozygous $Rh_0(D)$ red cells obtained from family studies.

4. The rh′(C) positive red cells took up only 70 to 73 per cent of the I^{131} anti-$Rh_0(D)$ that was bound to the rh′(C) negative cells. The decrease in quantity of bound I^{131} anti-$Rh_0(D)$ was uniform for both $Rh_0(D)$ genotypes, except for the cells homozygous for the rh′(C) antigen. This effect of the rh′(C) antigen on the $Rh_0(D)$ content may be apparent (immunochemical basis) or absolute (genetic mechanism).

5. Evidence for the heterogeneity of the $Rh_0(D)$ antigen was obtained. As a result the data presented apply only to the $Rh_0(D)$ antigen defined by the particular I^{131} anti-$Rh_0(D)$ used in this study.

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