REACTION OF I-131 TRACE LABELED HUMAN ANTI-RH$_0$(D) WITH RED CELLS

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REACTION OF I-131 TRACE LABELED HUMAN ANTI-Rh_0(D) WITH RED CELLS * †

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The reaction between red cell antigens and their corresponding isoantibodies is believed to occur in two distinct stages (1–3). The first stage involves the binding of the antibody to the antigen on the red cell stroma. It is generally conceded that this process represents a specific immunochemical reaction commonly referred to as "sensitization" of the red cell. The second stage which follows this reaction results in the visible agglutination of the red cell mass. The agglutination of red cells is a nonspecific process which can be significantly influenced by the nature of the suspending medium or by changes in the cell membrane. Both of these processes, "sensitization" and agglutination, are evident in the case of complete or saline active isoantibodies. With incomplete or blocking antibodies (4, 5), however, only the first stage is evident so that the isoantibody sensitizes the red cell but the reaction does not proceed to visible agglutination.

Laboratory detection of these antibodies consequently depends on enhancing the nonspecific agglutination stage. Enhancement of agglutination is commonly achieved through the use of three techniques: the use of large anisometric molecules in the cell suspending medium (6), enzyme digestion of the red cell stroma (7, 8), or use of the anti-globulin reaction (9). The primary or specific reaction between the red cell and its isoantibody, therefore, cannot be observed directly. Recognition of this reaction depends on an inference derived from the nonspecific reaction of agglutination.

The use of an I-131 labeled isoantibody (10) offers a means of studying directly the reaction of an incomplete isoantibody with red cells. Bours-

nell, Coombs and Rizk (11) appreciated the possibilities offered by the I-131 protein label and tagged human anti-Rh_0(D) serum. They were able to use the labeled isoantibody to estimate the number of Rh_0(D) sites and the Paul-Bunnell sites on the red cell, and more recently the antigen sites on the rabbit red cell (12). Melcher, Steinfeld and Reed (13), using both I-131 sheep red cell agglutinating rabbit serum and an I-131 incomplete red cell antibody, obtained from a patient with acquired hemolytic anemia, were able to show by zone electrophoresis that the red cell isoantibody activity was associated with the gamma globulin fraction.

Although these studies established the usefulness of I-131 labeled red cell isoantibodies, they were severely limited by the binding of nonspecific I-131 labeled proteins to the red cells. The problem of nonspecific binding of labeled proteins to red cells was re-examined in this report. The studies to be presented have shown that by the use of alcohol fractionation and elution techniques an I-131 trace labeled anti-Rh_0(D) can be obtained that reacts specifically with Rh_0(D) positive red cells.

MATERIALS AND METHODS

Fractionation of anti-Rh_0(D) plasma. High titered anti-Rh_0(D) plasma was obtained from a 30 year old white housewife who had been immunized to Rh_0(D) by eight pregnancies. The last nonviable delivery occurred in February, 1955, at which time she had both Fallopian tubes ligated. Her phenotype was A_2, rh(cde), and her serum contained an incomplete anti-Rh_0(D) with a titer of 64. She was injected intravenously with 1 ml. of type A_1, Rh_0(cDe) blood and phlebotomized 13 days later (14–16). The blood was collected by means of an ion-exchange plastic donor set that contained Dowex 50®. At this time her serum incomplete anti-Rh_0(D) titer was 8,192. The specificity of the antibody was anti-Rh_0(D) as determined by the anti-globulin reaction and a panel of known red cells (Panocell®, Knickerbocker, New York, N. Y.).

The calcium-free plasma was fractionated by a modification [1] of the Cohn ethanol method 10 procedure (17). In this modification Fraction III 2 is first removed by absorption onto $BaSO_4$, followed by precipitation of Fraction I at pH 6.8, $\Gamma/2 = 0.08$, 6 per cent ethanol at $-2°$ C. Fraction II + III 0,1,3 is then precipitated at pH 5.8, $\Gamma/2 = 0.04$, 19 per cent ethanol at $-5°$ C. Fraction II was then extracted from the II + III 0,1,3 paste with an acetate buffer at pH 4.9 containing 0.6 M glycine, $\Gamma/2 = 0.002$, 14 per cent ethanol at $-5°$ C. The remaining precipitate (Fraction III 0,1,3) was resuspended in 0.15 M phosphate buffer, pH 7.5. This fraction was subsequently designated A-18. In the other preparation, A-15, Fraction III-0 was extracted from the precipitated Fraction III 0,1,3 at pH 6.8, 0.6 M glycine, $\Gamma/2 = 0.005$, 15 per cent ethanol at $-5°$ C. The remaining paste (Fraction III 1,3) was dissolved in 0.15 M phosphate buffer, pH 7.5. Both preparations, A-18 and A-15, after fractionation were dialyzed against 0.15 M phosphate buffer, pH 7.5, preparatory to iodination.

The titer of anti-Rh_0(D) of the calcium-free plasma was 2,048 before fractionation. After fractionation and dialysis the anti-Rh_0(D) titer of A-18 was 1,024 and that of A-15 was 256. Anti-Rh_0(D) titers were determined using the method recommended by Dunsford and Bowley (1). Preparation A-15 contained 7.11 per cent and preparation A-18, 16.8 per cent of the original plasma nitrogen.

Iodination. The isoantibody-containing fractions were trace labeled with I-131 [2] by the tri-iodide method (I_3^-) at pH 7.5 in the presence of 0.15 M phosphate buffer (18, 19). The total nitrogen employed for iodination was 72 mg. for A-15 and 150 mg. for a A-18 at a concentration of 1.45 and 3.01 mg. nitrogen per ml., respectively. Unbound iodide-131 was removed by dialysis at 4° C. with a minimum of 20 L. of 0.15 M phosphate buffer at pH 7.5 containing 1 : 10,000 merthiolate over a 36 to 72 hour period. At the completion of dialysis the I-131 isoantibody preparation was clarified by centrifugation in a Spinco Model L Ultracentrifuge at 20,000 rpm for 10 minutes, maximum G 46,900.

Nonprotein-bound I-131 was determined by precipitating the I-131 proteins with 10 per cent cold trichloracetic acid in the presence of carrier iodide and carrier protein (human serum). The total quantity of iodine bound to the protein was calculated as described previously (18) using a molecular weight of 160,000. The properties of the two isoantibody preparations are presented in Table I.[3]

[1] The details of the modified method 10 were kindly provided by Dr. R. B. Pennell, of the Blood Characterization and Preservation Laboratory of the Protein Foundation, Inc., Jamaica Plain, Mass.

[2] Carrier-free I-131 was obtained from the Oak Ridge National Laboratory, Oak Ridge, Tenn.

[3] Note added in proof: Electrophoretic analysis of the I-131 anti-Rh_0 (D) preparations indicate that the fractions used in these studies all contained Fraction II + III instead of III alone.

Characterization of the I-131 anti-$Rh_0(D)$ fractions

Property	Preparation	
	A-15	A-18
Fraction	E III 1, 3	E III 0, 1, 3
% I-131 bound	10.9	13.2
M I/M globulin*	1.16	2.02
μc. I-131/μg. N†	0.94	0.75
% I-131 precipitable with trichloracetic acid	96.6	94.8
Titer before iodination	256	1,024
Titer after iodination	128	512
Nitrogen, μg. per ml.	1,080	2,270

* Calculated using a molecular weight of 160,000 for the total fraction iodinated.
† Activity at the time of iodination.

Determination of radioactivity. Measurement of I-131 gamma activity was carried out with a well-type scintillation detector using a thallium activated sodium iodide crystal (20) with four inches of lead shielding. The detector was coupled to a conventional scaler. The background counting rate for this detector, shielding and operating voltage (1,100 to 1,300) was 80 to 90 cpm. Detector efficiency was 7.03×10^5 cpm per μc. of I-131.[4] Sample and background counting rates were determined by using counting periods sufficient to maintain the total error below 5 per cent at the 0.05 level of significance (21). All samples had counting rates greater than three times background. Sample activity was maintained by volumetric dilution at a level which did not require correction for coincidence losses. Correction for radioactive decay was obviated by assaying a volumetrically diluted aliquot of the original I-131 isoantibody preparation with each sample throughout the course of the experiment. The radioactivity of all samples was converted to μg. nitrogen by use of the original I-131 reference sample. To check any variation in the efficiency of the counting equipment, the decay of the I-131 isoantibody standards throughout the course of the experiment was evaluated graphically.

Samples for radioactivity determination were prepared in 4 ml. capacity 15×45 mm. glass vials with Bakelite screw caps. Red cell suspensions were assayed by placing the Kahn reaction tubes directly in the well-counter.

Stroma preparation. Stroma preparations were derived from type O or A, Rh_0(D) positive red cells obtained from outdated units of whole blood. The stroma was prepared using the method described by Komninos and Rosenthal (22). The washed stroma was then lyophilized and maintained at $-20°$ C. until use. About 4 to 5 Gm. of stroma was obtained from 1 unit of whole blood with this technique

[4] Standardized against I-131 standards previously obtained from the National Bureau of Standards, Washington, D. C.

TABLE II

Properties of the I-131 anti-$Rh_0(D)$ eluates

Prepara-tion*	Elution procedure employed	T.C.A.† precipitable I-131	Titer	Nitrogen
				$\mu g./ml.$
A–15–1	Heat	87.2	1	0.399
A–15–2	Heat	89.5	4	0.604
A–15–3	Kidd method	80.9	Positive undiluted	0.201
A–18–1	Heat	84.7	Positive undiluted	0.076
A–18–2	Heat	80.4	Positive undiluted	0.316
A–18–3	Heat	90.3	1	0.332
A–18–4	Heat	94.9	1	0.380

* Alcohol fraction employed in obtaining eluate is indicated by the prefix of the preparation.
† Trichloracetic acid, 10 per cent.

Elution of I-131 anti-$Rh_0(D)$ from $Rh_0(D)$ stroma.
From 250 to 500 mg. of lyophilized red cell stroma equivalent to 25 to 50 ml. of whole blood was sensitized by incubation with the I-131 isoantibody preparations. The sensitized stroma was washed three times with 0.15 M phosphate buffer, pH 7.5, and I-131 anti-$Rh_0(D)$ was eluted from the washed sensitized stroma by the following modifications of Landsteiner and Miller's method (23). The eluting solution consisted of 3.0 ml. of 0.15 M phosphate buffer, pH 6.0, and either 0.5 ml. of Fraction III 1,3 (150 to 200 µg. of nitrogen) derived from type AB, $Rh_0(D)$ positive plasma, or in some cases of 1.0 ml. type AB, $Rh_0(D)$ positive plasma. When Fraction III 1,3 was used for the AB, $Rh_0(D)$ positive carrier, 0.25 ml. of 30 per cent commercial bovine albumin was used in addition to increase the carrier protein. The sensitized stroma was eluted repeatedly for five times with the same volume of eluting medium at 56° C. (about twice the volume of the packed stroma). The first eluate was derived from a 5 minute incubation and the remaining four from a 15 minute period of incubation. The eluates were separated from the stroma by centrifugation at 56° C. by operating the centrifuge (Serofuge®, Clay Adams) in a 56° C. oven. In some experiments, the clarified eluates were passed through a column of Amberlite IR-45® in a 10 ml. syringe to remove unbound iodide-131. In the remaining experiments dialysis of the pooled eluates against phosphate buffer was substituted for this step. In a few studies the method described by Kidd (24) was used to elute the I-131 isoantibody from the sensitized stroma (Table II). The eluates after treatment with either the ion exchange resin or after dialysis were clarified by 10 minutes' centrifugation at maximum G 46,900 in a Spinco Model L Ultracentrifuge and merthiolate was added to the clarified eluates to yield a final concentration of 1 : 10,000.

The biological activity of each eluate was determined by reacting each eluate with both $Rh_0(D)$ positive and negative red cells. The biologically active eluates were then pooled, dialyzed overnight against 0.15 M phosphate buffer, pH 6.5, and stored at 4° C. until used. The properties of the various eluates used in these studies are listed in Table II. Under these conditions of elution less than 2 per cent of the total I-131 added to the stroma was recovered in the eluates. A more detailed quantitative evaluation of the elution techniques used in these studies will be presented elsewhere.

Reaction of red cells with I-131 isoantibody preparations. Red cells were obtained from the donor population entering the Blood Bank. Venous blood was collected after phlebotomy was completed and heparin was used as the anti-coagulant. The cells were washed three times by centrifugation (International Clinical Centrifuge, Model CL) with a buffer wash solution consisting of 0.15 M phosphate buffer, pH 6.5, which contained by volume 10 per cent of a 0.16 M NaI, 0.016 M $NaHSO_3$ solution and 0.05 per cent of a commercial, 30 per cent bovine albumin. After the final wash the packed cells were resuspended in Alsever's solution equivalent to the original plasma volume and the red cells were stored at 4° C. Before use the cells were washed once with the buffer wash solution and resuspended in the same solution. All cells were used within five days after phlebotomy to avoid any changes induced in the red cell with these conditions of storage.

The cells were typed for the ABO and the Rh antigens using commercially obtained antisera. The techniques recommended by the manufacturer were employed and the results were controlled by using both positive and negative control cells.

Appropriate dilutions of red cells in the buffer wash solution were reacted in silicone-coated[5] Kahn serological tubes, 11 × 75 mm., with the I-131 isoantibody preparation. The red cell concentration used for most of these studies ranged from 2 to 10 volumes per cent. Cell concentration was determined by the microhematocrit method (25, 26). The capillary tubes were centrifuged (International Micro-hematocrit Centrifuge) for five minutes at maximum G 12,000. The red cells usually in a volume of 1 ml. were pipetted into the Kahn tubes containing the I-131 isoantibody with an Ostwald pipette. After addition of the red cells the tubes were stoppered with rubber stoppers, mixed and placed in a 37° C. water bath for one hour. The reaction tubes after incubation were centrifuged in 20 place bucketcups for three to five minutes at maximum G 1,650. The supernates were aspirated and saved for additional studies, and the cells washed twice with about 5 ml. of the buffer wash solution. The sensitized red cells were quantitatively transferred to a clean tube with Pasteur pipettes after the second wash, washed two additional times and then assayed for radioactivity. The determination of the red cell-bound I-131

TABLE III

Anti-Rh₀(D) specificity of I-131 isoantibody preparation *

Probable cell genotype†	Total cpm bound to cells	% I-131 bound	Nitrogen bound
			$\mu g. \times 10^{-2}$
R¹R² (CDe/cDE)	583	5.79	2.20
R²R² (cDE/cDE)	683	6.79	2.58
r'r (Cde/cde)	13	0.13	0.04
r"r (cdE/cde)	10	0.10	0.04
rr (cde/cde)	9	0.09	0.03

* Preparation A-18-1, 0.38 μg. nitrogen and 10,065 cpm in 5 ml. with 0.3 ml. of an 8 per cent cell suspension. Cells incubated one hour at 37° C. Each value represents the average of two determinations.

† Cells obtained from a panel of Group O cells prepared by Knickerbocker, New York, N. Y.

was carried out by placing the Kahn tube directly into the well of the scintillation counter.

Calculations and assumptions employed. The interpretation of the data obtained in these studies depends on a number of assumptions. Two of these involve the calculations used to derive the molar ratio of iodine to specific isoantibody. One of these assumptions has been the use of 160,000 for the molecular weight of the anti-Rh₀(D) and the other assumes that the iodine reacts equally and to the same extent with all the proteins in Fractions E-III 1,3 and E-III 0,1,3. Under these circumstances the iodine to nitrogen ratio of the total fraction would apply for all the proteins including the isoantibody. The finding that the iodine to nitrogen ratio of specific antibody is the same as that of the total gamma globulin fraction (18) offers support for this assumption. The stability of the iodine protein label *in vitro* has been confirmed by many studies (10). In view of this evidence the results obtained in these studies have been converted to nitrogen through the use of the iodine to nitrogen ratio of the total fraction that was iodinated. Although these studies do not provide direct experimental verification for these assumptions, valid and useful information can be obtained from these relative values (12).

A 1 ml. of a 10 per cent cell suspension was considered to be equivalent to 1.06×10^9 red cells. This value represents an approximation since red cells vary in volume from one individual to another. There is probably an even greater discrepancy when equating volumes per cent to number of red cells when the possible effects of storage on the red cell volume are considered.

Each sample of red cells was reacted with the I-131 isoantibody at least in duplicate and included a set of Rh₀(D) negative cells as a control. The I-131 bound to the Rh₀(D) positive cells was determined by subtracting the percentage of the total I-131 bound to the Rh₀(D) negative cells from that found on the Rh₀(D) positive cells. This corrected percentage of I-131 bound to the Rh₀(D) positive cells was then converted to nitrogen through the use of the I-131 to nitrogen ratio of the total isoantibody-containing fraction, A-18 or A-15. The I-131 taken up by Rh₀(D) negative cells was less than 3 per cent of that found on the Rh₀(D) positive cells.

Justification for this correction is based on both the magnitude of the Rh₀(D) negative cell value and the assumption that the I-131 bound nonspecifically to the Rh₀(D) negative cells is also taken up by the Rh₀(D) positive cells. The standard deviation of the overall determination of the amount of I-131 bound to a given red cell suspension was 2.6 to 1.5 per cent.

RESULTS

Iodine content and biological activity

There is no loss of biological activity with the introduction of one to two M iodine per M protein. The apparent decrease in titer after iodination (Table I) is due to dilution of the isoantibody which occurred during iodination.

I-131 binding by Rh₀(D) negative red cells

Rh₀(D) negative cells bind less than 2.5 per cent of the I-131 taken up by the Rh₀(D) positive cells. The Rh₀(D) specificity of the I-131 anti-

TABLE IV

Effect of antigen excess on quantity of I-131 bound to red cells *

Volume I-131 anti-Rh₀(D)	Total nitrogen added	Total cpm added to cells	Total cpm bound to Rh₀(D) positive cells	Total cpm bound to Rh₀(D) negative cells	Per cent I-131 bound			Nitrogen bound
					Rh₀(D) positive cells	Rh₀(D) negative cells	Corrected Rh₀(D) positive cells	
ml.	*μg.*							$\mu g. \times 10^{-2}$
1.0	0.076	2,265	245	4	10.82	0.18	10.64	0.81
2.0	0.153	4,530	442	12	9.76	0.26	9.50	1.45
3.0	0.229	6,795	554	17	8.15	0.25	7.90	1.81
4.0	0.306	9,060	642	6	7.09	0.07	7.02	2.15
5.0	0.382	11,330	660	16	5.83	0.14	5.69	2.17

* Preparation A-18-3 reacted with 0.2 ml. of a 20 per cent cell suspension at 37° C. for one hour. Probable genotypes: O-R¹r (CDe/cde) or R¹R⁰ (CDe/cDe) and O-rr (cde/cde).

TABLE V

Relationship of red cell concentration to antibody content *

Study	Total nitrogen added	Number of increments	Total time of incubation	Total cpm added to cells	Total cpm bound to Rh₀(D) positive cells	Total cpm bound to Rh₀(D) negative cells	Per cent I-131 bound			Nitrogen bound to cells
							Rh₀(D) positive cells	Rh₀(D) negative cells	Corrected Rh₀(D) positive cells	
no.	*μg.*									*μg.* $\times 10^{-2}$
I	0.60	1	60	12,750	847	24	6.64	0.19	6.45	3.9
II	1.20	2	120	25,960	1,675	· 59	6.45	0.23	6.22	7.5
III	1.81	3	180	37,080	2,013	71	5.43	0.19	5.24	9.5
IV	1.81	1	60	37,170	1,736	27	4.67	0.07	4.60	8.3

* All determinations in triplicate. Preparation A-15-2 with 1 ml. of a 10 per cent red cell suspension was incubated at 37° C. for the time period indicated in the table. Probable genotypes are O-R¹r (CDe/cde) or R¹R⁰ (CDe/cDe) and O-rr (cde/cde).

Rh₀(D) isoantibody preparation is indicated in Table III. Less than 2.5 per cent of the I-131 taken up by Rh₀(D) positive cells is bound by either rh'(C) or rh"(E) containing cells.

Relationship between isoantibody content and antigen sites

The effect of increasing the quantity of I-131 isoantibody on the total I-131 bound to Rh₀(D) positive cells is shown in Table IV. As the volume of isoantibody reacted with a constant volume of cells is increased, the I-131 (nitrogen) bound progressively increases until a maximum value is reached. The addition of more isoantibody to the cells at this point is without effect on the quantity of I-131 (nitrogen) bound to the red cells. In this particular experiment the addition of more

than 4 ml. of antibody resulted in antibody excess, so that saturation of all of the antigen sites had occurred. The reaction tubes with less than 4 ml. or 0.306 μg. of nitrogen are in the region of antigen excess where the number of antigen sites exceeds the available isoantibody.

The same relationship is also apparent from the data in Table V, Study I. This experiment in antigen excess, with only 0.6 μg. of isoantibody nitrogen, resulted in the binding of 0.039 μg. nitrogen. When three times the nitrogen was reacted with the same quantity of cells (Study IV) 0.083 μg. of nitrogen was bound. The addition of a second 0.6 μg. nitrogen increment of I-131 isoantibody to the cells of Study I resulted in the uptake of 0.075 μg. nitrogen. A third increment of I-131 isoantibody followed by a third incubation

TABLE VI

Specificity of the I-131 bound to red cells as determined by absorption studies

Source of supernate*	Total nitrogen added	Total cpm added to cells	Total cpm bound to Rh₀(D) positive cells	Total cpm bound to Rh₀(D) negative cells	Per cent I-131 bound			Nitrogen bound
					Rh₀(D) positive cells	Rh₀(D) negative cells	Corrected Rh₀(D) positive cells	
	μg.							*μg.* $\times 10^{-2}$
Rh₀(D) positive cells of Study I	0.47	8,931	70	15	0.78	0.17	0.61	0.3
Rh₀(D) negative cells of Study I	0.50	9,335	584	17	6.26	0.18	6.08	3.0
Rh₀(D) positive cells of Study IV	0.87	18,580	285†	12†	1.53	0.06	1.47	1.3
Rh₀(D) negative cells of Study IV	0.94	19,490	1,010†	19†	5.18	0.10	5.08	4.8

* These supernates were obtained from the studies tabulated in Table V. The supernates reacted with 1 ml. of a 10 per cent red cell suspension for one hour at 37° C. Probable genotypes are: O-R¹r (CDe/cde) or R¹R⁰ (CDe/cDe) and O-rr (cde/cde).
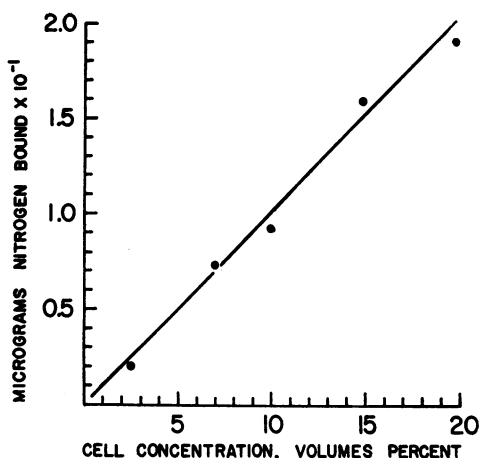† Duplicate determinations, all others in triplicate.

FIG. 1. EFFECT OF RED CELL CONCENTRATION ON THE QUANTITY OF I-131 BOUND TO THE RED CELL MASS

Each of the red cell concentrations indicated in the figure was suspended in 1 ml. buffer wash solution and incubated one hour at 37° C. with preparation A-15-1. Each value represents the average of two determinations. Probable genotypes: O-R¹r (CDe/cde) or R¹R° (CDe/cDe) and O-rr (cde/cde). Only the corrected $Rh_0(D)$ positive red cell values are plotted.

period resulted in a total of 0.095 μg. nitrogen bound to the red cells. These results indicate that the antigen sites on the red cell can be saturated by either reacting them with an excess of antibody or, if the reaction is in antigen excess, the antigen sites can be saturated by successive increments of antibody.

The reaction of the supernates obtained from Studies I and IV with $Rh_0(D)$ positive and negative red cells is shown in Table VI. The supernates recovered from the $Rh_0(D)$ negative red cells of Study I contained 0.03 μg. of specific nitrogen out of a total of 0.5 μg. or 6 per cent, which is comparable to the specific nitrogen content of the original preparation (6.5 per cent). The supernates obtained after reaction of the isoantibody with $Rh_0(D)$ positive red cells in Study I are almost free of I-131 isoantibody (0.003 μg. of nitrogen). The supernates from the $Rh_0(D)$ negative red cells of Study IV contain 0.048 μg. of specific nitrogen out of a total of 0.94 μg. of nitrogen, whereas the $Rh_0(D)$ positive red cell supernates from Study IV contain only 0.013 μg. of specific nitrogen. The antibody content of both supernates obtained from Study IV is inadequate to saturate all of the antigen sites present in 1 ml.

of a 10 per cent red cell suspension. About 50 per cent of the antibody required was present in the $Rh_0(D)$ negative red cell supernates, and about 14 per cent in the $Rh_0(D)$ positive red cell supernates. The presence of antibody in the $Rh_0(D)$ positive red cell supernates of Study IV indicates that this study was carried out in antibody excess. The relationship between the I-131 bound to the red cell mass and the concentration of red cells plotted as volumes per cent is indicated in Figure 1. A direct proportionality exists between the amount of I-131 bound to the red cells and the concentration of red cells. The nitrogen bound to 1 ml. of a 10 per cent red cell suspension as estimated from these data is 0.092 μg.

Determination of the rate of reaction between anti-$Rh_0(D)$ and $Rh_0(D)$ positive red cells

The amount of I-131 bound to red cells as a function of time at 37° C. is shown in Figure 2. The curve obtained is an exponential growth curve that is consistent with a first order reaction, which follows the equation $A = A_0(1-e^{-kt})$, where A is the μg. nitrogen bound at time t, A_0 is the μg. nitrogen required to saturate all of the antigen sites and k is the reaction constant. A_0 for the cells used was 0.053 μg. nitrogen and k has a value of 5.78 per cent per minute or a half-time of 12 minutes.
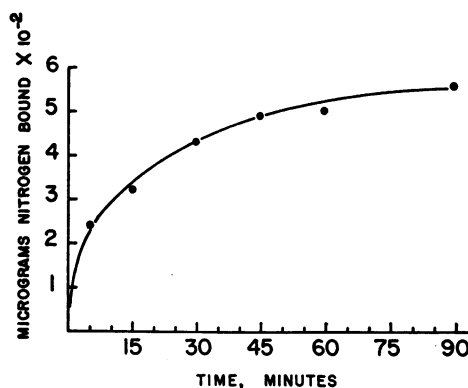

FIG. 2. EFFECT OF INCUBATION TIME ON THE QUANTITY OF I-131 BOUND TO THE RED CELLS

A 5 per cent red cell suspension in 1 ml. buffer wash solution was incubated at 37° C. with 2 ml. of preparation A-15-1, 0.80 μg. N, for the time indicated on the curve. Each value represents the average of three determinations. Probable genotypes: O-R¹r (CDe/cde) or r'R° (Cde/cDe) and O-rr (cde/cde). Only the corrected $Rh_0(D)$ positive red cell values are plotted.

FIG. 3. EFFECT OF TEMPERATURE ON THE QUANTITY OF
I-131 BOUND TO RED CELLS

Preparation A-15-3, 0.804 µg. nitrogen in 4 ml., was added to 1 ml. of a 5 per cent red cell suspension in buffer wash solution. Red cells were incubated one hour at the temperature indicated on the curve. Each value represents the average of two determinations. Probable genotypes: O-R¹r (CDe/cde) or R¹R° (CDe/cDe) and O-rr (cde/cde). Only corrected Rh₀(D) positive red cell values are plotted.
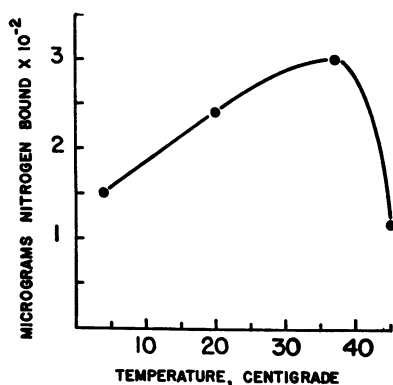
Temperature and pH dependence of the reaction

The effect of temperature on the reaction between Rh₀(D) positive red cells and I-131 anti-Rh₀(D) is shown in Figure 3. The amount of I-131 bound to the red cells increases progressively from 4° C. to a maximum at 37° C. and then decreases markedly beyond 37° C.

The effect of pH on the quantity of I-131 bound to red cells is indicated in Figure 4. The maximum quantity of I-131 bound to the red cells occurs within a pH range of 6.5 to 7.0. On the acid side of this optimal pH region at pH 6.0 there is marked decrease in the I-131 taken up by the
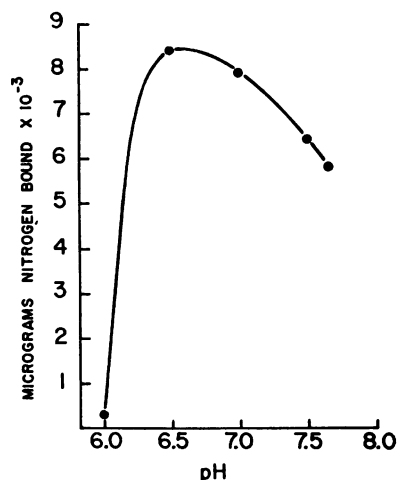

FIG. 4. EFFECT OF pH ON THE QUANTITY OF I-131 BOUND
TO RED CELLS

Preparation A-18-1, 0.38 µg. of nitrogen in 5 ml., was added to 0.2 ml. of a 10 per cent red cell suspension. Both cells and the isoantibody preparation were made up in phosphate buffer with the pH indicated on the curve. Cells were incubated one hour at 37° C. Each value represents the average of two determinations. Probable genotypes: O-R°r (cDe/cde) or R°R° (cDe/cDe) and O-rr (cde/cde). Only corrected Rh₀(D) positive red cell values are plotted.

red cells. This effect seems to be associated with an increase in the I-131 bound to the Rh₀(D) negative red cells. On the alkaline side of the optimal pH range there is a more gradual decrease in the quantity of I-131 bound to the red cells.

Inhibition studies

Specific blocking or inhibition of the I-131 bound to Rh₀(D) positive red cells occurs when the cells

TABLE VII

Effect of pretreatment with unlabeled anti-Rh₀(D) on the quantity of I-131 bound to red cells *

Pretreatment	Total cpm bound to Rh₀(D) positive cells	Total cpm bound to Rh₀(D) negative cells	Per cent I-131 bound			Nitrogen bound
			Rh₀(D) positive cells	Rh₀(D) negative cells	Corrected Rh₀(D) positive cells	
						µg. ×10⁻³
Anti-Rh₀(D) serum	21	7	0.10	0.03	0.07	1.1
O-Rh₀(D) negative serum	411†	9†	1.98	0.04	1.94	30.6
PO₄ buffer control	374	8	1.80	0.04	1.76	27.7

* Cells consisted of 0.2 ml. of a 20 per cent cell suspension, incubated at 37° C. for one hour with sera indicated in column one, washed four times with buffer wash solution and reincubated with 5 ml. of I-131 anti-Rh₀(D) preparation A-18-2, for one hour at 37° C. This volume of A-18-2 contained 1.575 µg. nitrogen and 20,740 counts per minute. Probable genotypes are: O-R¹R¹ (CDe/CDe) or R¹r' (CDe/Cde) and O-rr (cde/cde).
† Duplicate determinations; all others in triplicate.

TABLE VIII

Effect of heat on the ability of I-131 anti-Rh$_0$(D) to bind to red cells *

Treatment of the I-131 anti-Rh$_0$(D) preparation	Total μg. nitrogen added to cells	Total cpm added to cells	Total cpm bound to Rh$_0$(D) positive cells	Total cpm bound to Rh$_0$(D) negative cells	Per cent I-131 bound			Nitrogen bound
					Rh$_0$(D) positive cells	Rh$_0$(D) negative cells	Corrected Rh$_0$(D) positive cells	
								$\mu g. \times 10^{-2}$
Unheated, control	1.32	10,450	516	2†	4.94	0.02	4.92	6.49
Heated 60° C. for 30 minutes	1.33	10,540	502	2†	4.76	0.02	4.74	6.31
Heated 70° C. for 30 minutes	1.27	10,020	303	2†	3.02	0.02	3.00	3.81

* Cells consisted of 1 ml. of a 5 per cent suspension incubated with 4 ml. of preparation A-18-3 for one hour at 37° C.   Probable genotypes are: O-R²r (cDE/cde) or R²R⁰ (cDE/cDe) and O-rr (cde/cde).

† Duplicate determinations; all others in triplicate.

are pretreated with a serum containing anti-Rh$_0$(D) (Table VII). The red cells in this experiment were reacted at 37° C. for one hour with an unlabeled high-titered anti-Rh$_0$(D) serum, a nonantibody containing O-Rh$_0$(D) negative serum and phosphate buffer. The anti-Rh$_0$(D) containing serum employed in this study was obtained from the same donor who provided the plasma that was used for preparing the I-131 anti-Rh$_0$(D). Following the reaction of the cells with these three different preparations the cells were washed four times and reincubated with the I-131 anti-Rh$_0$(D) preparation. The cells treated with the serum containing unlabeled anti-Rh$_0$(D) took up 0.0011 μg. of nitrogen or less than 4 per cent of the nitrogen bound to the control cells.

Effect of heat on the biological activity of the I-131 anti-Rh$_0$(D)

The effect of heat on the biological activity of the I-131 anti-Rh$_0$(D) was studied by heating two aliquots of the preparation, one at 60° C. and the other at 70° C. for 30 minutes. The heated preparations were then reacted with red cells (Table VIII). The biological activity of the preparation heated for 30 minutes at 60° C. was unaffected, whereas the preparation heated for 30 minutes at 70° C. retained only about 60 per cent of the activity found in the unheated preparation. For purposes of interpretation it should be noted that even the preparation labeled unheated has been subjected to 56° C. for about 70 minutes as a result of the elution technique that was used to ob-

TABLE IX

Effect of hemolysis on the I-131 bound to red cells *

Preparation assayed†	Total μg. nitrogen added to cells	Total cpm added to cells	Total cpm bound to Rh$_0$(D) positive cells	Total cpm bound to Rh$_0$(D) negative cells	Per cent I-131 bound			Nitrogen bound
					Rh$_0$(D) positive cells	Rh$_0$(D) negative cells	Corrected Rh$_0$(D) positive cells	
								$\mu g. \times 10^{-2}$
I. Red cells	1.52	13,050	742	35	5.69	0.08	5.61	8.52
I. Stroma of above cells, centrifugation 15 min. at maximum 49,600 G	1.52	11,990‡	674‡	0‡	5.62	0.00	5.62	8.54
II. Red cells	3.04	21,890	1,060	13	4.84	0.06	4.78	14.5
II. Stroma of above cells, centrifugation 5 min. at maximum 1,650 G	3.04	20,330‡	585‡	10‡	2.88	0.05	2.83	8.60

* Values in table represent the average of duplicate determinations.

† Preparation A-18-4, 4 ml. for Study I and 8 ml. for Study II reacted with 1 ml. of a 10 per cent red cell suspension for one hour at 37° C.   Study I was in antigen excess.

‡ Difference between these values and the red cell values is due to decay that occurred during the time that the stroma was prepared.

tain these preparations. Consequently, a truly unheated control was not used for this study.

Effect of hemolysis on red cell-bound I-131

Red cells were hemolyzed with distilled water after they had been sensitized with I-131 anti-$Rh_0(D)$ (Table IX). In one experiment the red cell stroma was recovered by centrifugation in a Spinco Model L Ultracentrifuge for 15 minutes at maximum G 49,600 and in the other experiment the stroma was obtained by centrifugation for 5 minutes at maximum G 1,650 in a clinical centrifuge. With high-speed centrifugation the I-131 associated with the intact red cells was recovered completely in the centrifuged red cell stroma. With low-speed centrifugation only about 60 per cent of the red cell I-131 was found in the stroma. Failure to recover all the I-131 in the red cell stroma following low-speed centrifugation is probably due to incomplete recovery of the red cell stroma under these conditions of centrifugation.

DISCUSSION

The interpretation of the data obtained in these studies is based on the specificity of the I-131 bound to red cells (11–13). The effective exploitation of I-131 red cell isoantibodies depends on unequivocal evidence that the I-131 taken up by the red cells represents the specific isoantibody and conforms with the known behavior of these antibodies.

The first step in achieving this goal has been the recognition that red cell isoantibodies even in high-titered sera are present essentially in trace quantities, constituting probably less than 1 per cent of the plasma proteins (27). As a result efforts to concentrate the isoantibody by fractionation of the plasma are required. Even the concentration of the isoantibody by ethanol fractionation failed to yield a preparation which was effective following iodination. Only with further purification achieved by eluting the I-131 isoantibody from sensitized red cell stroma could preparations of sufficient biological activity be obtained.

A number of criteria have been employed to evaluate the nature and specificity of the I-131 preparations obtained with these techniques. The

simplest procedure was to use $Rh_0(D)$ negative cells with each experiment to evaluate the I-131 bound to red cells nonspecifically. Less than 3 per cent of the I-131 bound to the $Rh_0(D)$ positive red cells was found on the $Rh_0(D)$ negative red cells with the preparations used in these studies. In addition, both rh'(C) and rh''(E) red cells when reacted with the I-131 anti-$Rh_0(D)$ bound less than 3 per cent of the I-131 found on the $Rh_0(D)$ positive red cells. These findings indicate that the specificity of the I-131 isoantibody preparation is confined to the $Rh_0(D)$ antigen.

The quantitative immunochemical nature of the reaction between the $Rh_0(D)$ antigen and its isoantibody is evident from a number of the studies described.

The direct proportionality between the quantity of I-131 bound to the red cell mass and concentration of red cells (Figure 1) is consistent with the behavior of an antigen-antibody reaction. When an excess of red cells was used so that the available antigen sites exceeded the amount of antibody present, i.e., antigen excess (Tables IV and V), the quantity of nitrogen bound to the red cells was less than that bound by the cells reacted at equivalence. Cells reacted under conditions of antigen excess and only partially saturated with antibody took up additional nitrogen when successively reacted with more antibody, until all the antigen sites were saturated. Absorption of the I-131 anti-$Rh_0(D)$ with $Rh_0(D)$ positive red cells quantitatively removed the I-131 capable of being bound to red cells.

Supernates obtained from reactions carried out at equivalence or antibody excess contained demonstrable quantities of I-131 that could be specifically bound to $Rh_0(D)$ positive red cells (Table VI). In contrast absorption of the I-131 isoantibody preparation with $Rh_0(D)$ negative red cells had no effect on the content of I-131 that was bound to $Rh_0(D)$ positive red cells. These quantitative relationships between the $Rh_0(D)$ red cell antigen and the I-131 isoantibody confirm inferences made previously (28) with unlabeled systems.

The specific nature of the I-131 bound to the $Rh_0(D)$ positive red cells is supported by the inhibition or blocking studies (Table VII). In this experiment the red cell uptake of I-131 was com-

pletely blocked by treating the $Rh_0(D)$ positive red cells with a serum containing unlabeled anti-$Rh_0(D)$. These results provide direct evidence for the identity of the anti-$Rh_0(D)$ with the I-131 bound to the red cell.

Additional evidence for the specificity of the I-131 bound to red cells is provided by the reaction rate studies (Figure 2). The reaction between red cells and I-131 anti-$Rh_0(D)$ can be described as a first order reaction with a half-time of 12 minutes. The conventional incubation time of 60 minutes would result in saturation of 97 per cent of the available antigen sites when this constant is used in the calculation. It is apparent that the value obtained for the reaction constant using I-131 anti-$Rh_0(D)$ is consistent with the incubation time routinely employed for the detection of incomplete anti-$Rh_0(D)$.

The optimum temperature for the reaction between I-131 anti-$Rh_0(D)$ and red cells as determined by the maximum red cell uptake of I-131 occurred at 37° C. (Figure 3). This temperature is universally recognized as the optimum temperature for the detection of immune red cell isoantibodies (1–3). The marked drop in I-131 anti-$Rh_0(D)$ bound at 45° C. would also be expected in view of the widespread use of heat for the elution of isoantibodies from sensitized red cells (22, 23).

The pH dependence of the reaction (Figure 4) with the optimum between pH 6.5 to 7.0 confirms the studies of Carter (29) who found maximum Rh isoantibody titers at a pH of 7.0. The decrease on the acid side of the pH optimum probably is due in part to changes in the red cell which result in an increased susceptibility to hemolysis. If hemolysis occurs, loss of the stroma during conventional centrifugation could account for the decrease in the amount of I-131 bound to the red cells at pH 6.0.

Thermal stability is another property which has been used to characterize incomplete isoantibodies (1–3, 9). Heating the I-131 anti-$Rh_0(D)$ for 30 minutes at 60° C. (Table VIII) produced no decrease in the quantity of I-131 bound to $Rh_0(D)$ positive red cells. Even 30 minutes at a temperature of 70° C. did not completely abolish the ability of the I-131 anti-$Rh_0(D)$ to sensitize $Rh_0(D)$ positive red cells.

The classical interpretation of the red cell-iso-

antibody reaction postulates that the isoantibody is attached to the red cell stroma. Support for this view stems from the observation that antibodies can be eluted from stroma that has been obtained by hemolyzing antibody-coated red cells (22–24). The results of these studies with I-131 anti-$Rh_0(D)$ are consistent with this interpretation of the red cell-isoantibody reaction. Complete recovery of all the I-131 anti-$Rh_0(D)$ bound to red cells was obtained in the red cell stroma after hemolysis (Table IX). Failure to obtain all of the I-131 in the stroma following low-speed centrifugation is probably due to loss of some of the stroma with these conditions of centrifugation.

An estimate of the number of antigen sites on each red cell can be made using these data if the assumptions discussed previously are granted. At the present time no value has been assigned to the molecular weight of the $Rh_0(D)$ isoantibody. The value 1.6×10^5 for the molecular weight (30) has therefore been used in these calculations. The other assumption is that the iodine to nitrogen ratio of the specific antibody is the same as that of the total alcohol fraction. The agreement in the quantity of nitrogen bound to $Rh_0(D)$ positive red cells between two different fractions, A-18 and A-15, that differed both in nitrogen content and in protein content (A-18 contained the lipoproteins) suggests that the iodine content of all the proteins in the fraction is proportional to the protein nitrogen.

An additional approximation which is necessary to calculate the number of antigen sites on a red cell assumes that the red cell volume is directly proportional to the red cell number. Obviously this approximation will not hold up in comparing red cells of one individual to those of another. This assumption is probably even less reliable when applied to red cells that have been stored *in vitro* in view of the tendency of red cells to undergo changes in volume under these conditions.

The maximum number of $Rh_0(D)$ sites on each red cell obtained by using these assumptions is 2,000 to 3,000. The calculations involve converting the nitrogen bound to number of molecules by means of the molecular weight and Avogadro's number. The number of molecules bound to the cell mass is then divided by the estimated number of cells, 1.064×10^9 in 1 ml. of a 10 per cent cell suspension. This value is about one-half of that

reported previously (11). The discrepancy is probably related to the fact that these authors iodinated whole serum rather than plasma protein fractions.

The data, interestingly enough, also suggest that the $Rh_0(D)$ positive red cells can be segregated into two groups in terms of the quantity of nitrogen bound. About 60 per cent of the cells used in these studies take up about 0.10 μg. of nitrogen and the remaining 40 per cent of the cells, about 0.05 μg. of nitrogen. The difference between these two populations of cells may be related to homozygosity for $Rh_0(D)$ in the cells that bound 0.10 μg. of nitrogen and to heterozygosity for $Rh_0(D)$ in the cells that bound in the range of 0.05 μg. of nitrogen. More definitive studies will be required to firmly establish the one to one correspondence between genetic constitution and the number of antigen sites on the red cell, in view of the assumptions that were used in these calculations.

The results obtained in these studies with I-131 anti-$Rh_0(D)$ confirm and provide direct evidence for the classical interpretation of the reaction between red cells and isoantibodies. The I-131 labeled anti-$Rh_0(D)$ has enabled direct observation and quantitation of the physical attachment of the antibody to the red cell. These observations lend support to the concept that the "sensitization" of the red cell is independent of agglutination. The nature of the attachment of the antibody to the red cell is consistent with the concept that the red cell has discrete antigen sites on the stroma and that the antibody is firmly and quantitatively bound to these sites. Extension of these techniques to other red cell antigens should provide a better understanding of many problems in the field of hematology, blood banking and human genetics.

SUMMARY

1. Trace labeled I-131 anti-$Rh_0(D)$ was prepared by iodinating isoantibody-containing fractions obtained by alcohol fractionation of high-titered anti-$Rh_0(D)$ plasma. An I-131 anti-$Rh_0(D)$ was prepared by eluting the anti-$Rh_0(D)$ from red cell stroma sensitized with the I-131 alcohol fractions (E-III 0,1,3, and E-III 1,3).

2. The evidence for the specificity of the I-131 bound to red cells following the use of these preparations consists of the following observations:

a. Similarly treated $Rh_0(D)$ negative red cells bind less than 3 per cent of the I-131 taken up by the $Rh_0(D)$ positive red cells.

b. Selective removal or absorption of the I-131 anti-$Rh_0(D)$ by $Rh_0(D)$ positive red cells and failure of $Rh_0(D)$ negative red cells to absorb or remove the I-131 anti-$Rh_0(D)$ activity has been demonstrated.

c. A direct proportionality exists between the amount of I-131 bound to the $Rh_0(D)$ positive red cells and the concentration of red cells.

d. Complete inhibition of the binding of I-131 occurs when the red cells have been pretreated (sensitized) with unlabeled anti-$Rh_0(D)$ serum.

e. The reaction between I-131 anti-$Rh_0(D)$ and $Rh_0(D)$ positive red cells has been characterized as a first order reaction with a rate constant of 5.78 per cent per minute.

f. The temperature and pH optima as well as the thermal stability of the I-131 anti-$Rh_0(D)$ preparation are similar to those found with the native anti-$Rh_0(D)$.

g. The I-131 bound to red cells has been quantitatively recovered in the stroma after hemolysis of I-131 sensitized red cells.

3. An estimate made from these data indicates that there are 2,000 to 3,000 $Rh_0(D)$ antigen sites on each red cell.

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