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Interaction of IgG and its Fragments with Red Cells

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Summary. The red cell uptake of ^{131}I following incubation of red cells with [^{131}I] IgG prepared from normal donors was shown to be IgG and not trace contaminants such as transferrin, lipids or iodide. The criteria used were immunodiffusion, DEAE chromatography, gel filtration and exchange with unlabelled lipoproteins and plasma. The uptake of [^{131}I]IgG was pH and ionic strength dependent and was influenced by the proportion of cells to IgG present during the reaction. With constant cell concentration the uptake of [^{131}I]IgG increased progressively as more IgG was added to the cells and approached an asymptotic value suggesting that there was saturation of red cell binding sites. When the IgG concentration was kept constant the uptake of IgG was inversely proportional to the red cell concentration. No difference in the molar binding of IgG, Fab or Fc was found indicating that the non-antibody binding of IgG does not preferentially involve any part of the IgG molecule. The molar quantities of carefully prepared [^{131}I]IgG bound to red cells were similar to those obtained with [^{131}I]BSA. The non-antibody red cell binding of IgG was contrasted with the antibody type of IgG binding.

INTRODUCTION

Red cells suspended in a protein solution appear to take up or bind on their surfaces small amounts of protein which cannot be readily removed by washing. Recognition of this phenomenon is due in large part to the use of radioactive iodine labelled proteins which permit the investigator to trace minute amounts of proteins. In their studies with ^{131}I-labelled red cell antibodies Boursnell, Coombs and Rizk (1953) showed that $Rh_0(D)$ negative cells would take up almost as much ^{131}I as did the $Rh_0(D)$ positive cells. Furthermore, the non-specific cell-bound [^{131}I]IgG behaved anomalously, in that cells coated in this fashion with IgG did not agglutinate when anti-globulin sera were added to the red cells. Other investigators (Pirofsky, Cordova and Imel, 1962; Costea, Schwartz, Constantoulakis and Dameshek, 1962) were able to show that this 'non-specific', non-antibody type of binding occurred with labelled γ-globulin fractions as well as with labelled whole serum.

An obvious explanation for the failure of the red cell bound radioactive material to participate in the anti-globulin reaction is that it is not γ-globulin. Hughes-Jones and Gardner (1962) have suggested that the radioactivity bound to the red cell under these conditions may be due to ^{131}I-labelled lipids which exchange with the lipids in the red cell stroma. Another possibility that has been considered is that the cell bound radioactivity is due to the exchange of inorganic ^{131}I with intracellular red cell anions. Finally, it is conceivable that if transferrin is a contaminant in the labelled fraction, the red cell uptake

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of radioactivity may represent the binding of labelled transferrin to reticulocytes and young red cells (Jandl, Inman, Simmons and Allen, 1959; Jandl and Katz, 1963; Morgan and Laurell, 1963; Morgan, 1964).

In the present study an attempt was made to define the nature of the labelled material bound to the red cell and to quantitate and evaluate the factors that influence this uptake. In addition, the 'non-specific' binding of proteins to red cells was contrasted with the 'specific' antibody type of binding such as is observed with anti-$Rh_0(D)$ and $Rh_0(D)$ positive red cells.

MATERIALS AND METHODS

Preparation of human IgG

IgG was prepared from normal fresh blood of six different $Rh_0(D)$ positive donors collected in acid–citrate–dextrose solution. The unhaemolysed plasma was defibrinated at 37° by adding to 250 ml plasma 60 units of bovine thrombin in 5 ml 1·0 M $CaCl_2$. A crude IgG fraction was obtained by removing the lipoproteins and macroglobulins by differential density centrifugation. The density of the serum was adjusted to 1·065 by adding 2·65 M NaCl and 2·95 M KBr. The mixture was then centrifuged at g_{max} = 135,000 for 16 hours at 5°. The coloured intermediate layer containing the IgG was recovered after removing the top layer containing the low-density lipoproteins. The IgG containing fraction was equilibrated by dialysis with 0·01 M phosphate buffer, pH 8·0, containing 0·02 per cent NaN_3 and chromatographed twice on DEAE-cellulose (Whatman) equilibrated against the same buffer. The IgG-containing eluates were concentrated by pervaporation and in a few cases by lyophilization.

For some experiments IgG was prepared from a lyophilized Cohn Fraction II which was further purified by DEAE chromatography as described above.

Preparation of papain fragments of IgG

The purified Cohn Fraction II preparation of human IgG was used for digestion. The IgG was digested with papain using the method of Porter (1959) and the papain fragments were isolated according to the procedure of Franklin (1960). Non-digested IgG and small molecular weight polypeptides were removed from the A, B and C fragments by chromatography on Sephadex G-200 (Tan and Epstein, 1963).

Preparation of lipoproteins

The low density fraction of the β-lipoproteins was obtained from the supernatant layer following differential density centrifugation of serum adjusted to a density of 1·065 as described above. A high density fraction of α-lipoproteins was obtained by raising the serum density to 1·210.

Protein determinations were done with both the micro-Kjeldahl technique and the biuret method as described by Kabat and Mayer (1961). Ultracentrifugal analyses were carried out at 59,720 rev/min at 25° using a protein concentration of 0·5–1·5 mg/ml in a 0·15 M phosphate–saline buffer.

Immunological procedure

Immunoelectrophoresis was carried out as described by Scheidegger (1955) and double diffusion by the technique of Ouchterlony (1953).

The following antisera were used: equine anti-whole human serum (Pasteur Institute, Lot Nos. 223 and 419), rabbit anti-β, anti-α_1 and anti-α_2 human lipoproteins (Behring-Werke, Marburg), rabbit anti-human transferrin (purified using transferrin coupled to a *p*-aminobenzoic acid cellulose column by Dr C. Bron, Institute of Biochemistry, University of Lausanne) and sheep anti-human IgG (containing 8·6 mg precipitating antibody protein per ml). Rabbit anti-human Fab was prepared by immunizing rabbits with the Fab fraction obtained as described above using Freund's adjuvant.

The anti-globulin test was performed according to Dunsford and Grant (1959).

Iodination of proteins

The proteins were labelled with ^{131}I using the chloramine-T procedure described by Bocci (1964) with the following modifications. Two to 4 mc of ^{131}I were used to label 50–80 mg of protein and sufficient carrier NaI was added to introduce 2–3 atoms of iodine per molecule of protein. Forty micrograms of chloramine-T were added to each milligram of protein in 0·15 M, pH 7·4, phosphate buffer at room temperature and the reaction was stopped after 30 minutes by the addition of 40 μg of sodium metabisulphite per milligram of protein. ^{131}I was removed by exhaustive dialysis against phosphate saline buffer, pH 7·3, 0·15 M at 4°.

The non-protein ^{131}I was determined by precipitation of the IgG with 10 per cent trichloracetic acid and by paper electrophoresis.

Incubation of red cells with ^{131}I proteins

Erythrocytes collected in Alsever's solution were used for tests within a 3- to 10-day period. The erythrocytes, prior to use, were washed with isotonic phosphate buffered saline, pH 7·3, and suspended in the same buffer to give a 10 per cent suspension. The number of red cells per millilitre was determined by electronic counting.

The red cells were incubated with the [^{131}I]IgG at 37° for 45 minutes in 1 × 10 cm glass tubes. The final volume of all tests was 1 ml. After reaction the cells were washed by centrifugation g_{max} = 400 for 5 minutes. Washing was done at 22° by aspirating 0·9 ml of the supernatant after centrifugation and re-suspending the cells in 2 ml of the buffer wash solution. A constant volume (1·9 ml) of supernatant was aspirated after each wash. The number of washes is indicated for each experiment and was varied from three to fifteen. After the cells were washed the ^{131}I bound to the red cell button was determined by scintillation counting.

The quantity of [^{131}I]protein adsorbed to the glass reaction tube after incubation and three washes was evaluated in some experiments by transferring the red cell button to a fresh tube and re-determining the radioactivity associated with the red cell button. In these experiments there was no significant difference in the cell bound radioactivity between the one tube procedure and the two tube transfer procedure. To avoid loss of red cells during transfer most of the experiments were carried out using a one tube procedure.

Red cells were separated according to their *in vivo* age using the method of Rigas and Koler (1961).

Determination of radioactivity

The ^{131}I content of the different samples was measured by γ-ray spectrometry by standard techniques. All samples had counting rates greater than ten times background.

Calculations

The ^{131}I bound to the red cells was converted to micrograms of IgG protein by using the iodine to nitrogen ratio of the labelled IgG fraction. The number of IgG molecules bound to a red cell represents an average value and is based on the assumption that the cell bound IgG is evenly distributed on the red cells present in the suspension. A molecular weight of 160,000 for IgG was used for the calculations. The standard error of the mean was calculated and the student's *t*-test was used to determine the significance of any difference observed.

RESULTS

CHARACTERIZATION OF THE IgG FRACTIONS AND IgG FRAGMENTS

All the IgG fractions at a concentration of 8 mg/ml showed a single line on immunoelectrophoresis when tested with a potent horse anti-human serum.

Anti-transferrin and anti-α_1-, α_2- and β-lipoprotein sera potent enough to give precipitation arcs with 0·02 per cent solutions of the corresponding antigens in the double immunodiffusion techniques did not give any precipitation with a 10 per cent IgG solution. By this criterion the IgG preparations contained less than 0·2 per cent of transferrin or lipoproteins.

The behaviour of the [^{131}I]IgG preparations on DEAE-cellulose chromatography was evaluated by mixing the [^{131}I]IgG with unlabelled serum equilibrated with 0·01 M phosphate buffer at pH 8·0. The serum provided a five-fold excess of unlabelled IgG. The ^{131}I content of the eluted fractions was compared with the nitrogen content of the fractions as measured by optical density. There was a correspondence between the radioactivity and nitrogen content of each fraction, except for a minimal shift of the radioactivity curve to the right which may be due to a charge modification resulting from the formation of iodotyrosine residues.

No evidence of aggregation could be detected following gel filtration of the freshly prepared [^{131}I]IgG preparations on Sephadex G-150 and G-200. The ^{131}I content of the eluted fractions described a symmetrical peak identical to that observed following gel filtration of unlabelled IgG.

Less than 1 per cent of the total ^{131}I in the IgG preparations was non-protein bound. In analytical ultracentrifugation the IgG gave a sharp symmetrical peak with a sedimentation coefficient of 6·6S.

The IgG papain fragments were homogeneous by the criteria of immunoelectrophoresis, double immunodiffusion, ultracentrifugation and absorption, using the antiglobulin reaction as described previously (Grob, Isliker and Webb, 1966).

EXCHANGE OF ^{131}I OF THE IgG FRACTIONS WITH LIPOPROTEINS

One hundred micrograms of [^{131}I]IgG containing a total of $2{\cdot}36 \times 10^5$ counts/min were mixed with saline; with aliquots of fresh serum adjusted to a density of 1·065 and 1·210; with low density lipoproteins adjusted to a density of 1·065 and with high density lipoproteins adjusted to a density of 1·210. After adjusting the volume to 11·8 ml, the tubes were centrifuged at $g_{max} = 105{,}000$ for 21 hours. The ^{131}I content of the upper 0·5 ml lipoprotein containing layer was measured. After centrifugation none of the lipoprotein or serum containing tubes had more than 0·2 per cent of the total ^{131}I in the upper 0·5 ml

fraction. The upper 0·5 ml fraction should contain 4·2 per cent of the total radioactivity, if no stratification of the ^{131}I was produced by flotation or sedimentation during centrifugation.

EXCHANGE OF ^{131}I WITH INTRACELLULAR RED CELL ANIONS

A 10 per cent red cell suspension, 0·1ml, was incubated with 700 μg of an [^{131}I]IgG preparation that had not been dialysed after iodination. The undialysed IgG contained 30 per cent of the total radioactivity in the form of free ^{131}I. The same IgG preparation following dialysis was used as a control. After incubation at 37° for 1 hour and six washes, $0{\cdot}80 \pm 0{\cdot}011$ per cent of the ^{131}I from the dialysed preparation was fixed to the red cells and $0{\cdot}95 \pm 0{\cdot}013$ per cent of the ^{131}I from the undialysed fraction was cell bound.

RELATIONSHIP BETWEEN QUANTITY OF IgG ADDED AND RED CELL UPTAKE OF IgG

The quantity of IgG bound to a constant number of red cells as the concentration of added IgG is increased is shown in Fig. 1. The red cell fixation was proportional to the

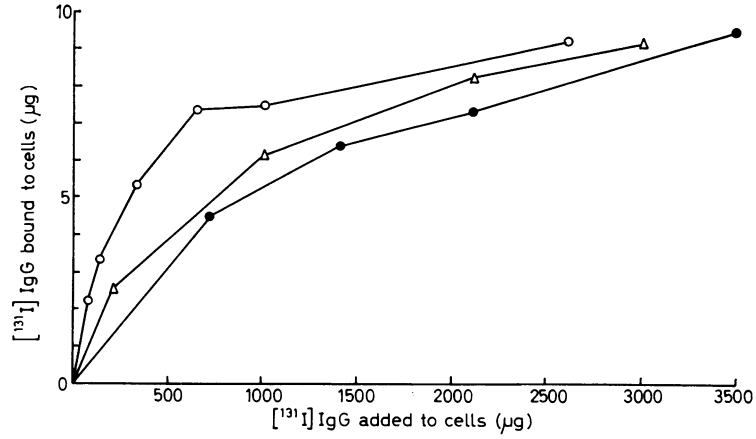

FIG. 1. Relationship between quantity of IgG added and red cell uptake of IgG. The [^{131}I]IgG was added to a constant number of red cells, 0·1 ml of a 10 per cent suspension. The incubation was carried out at 37° for 1 hour at pH 7·3, 0·15 M, ionic strength 0·16. Total volume was 1·0 ml and the cell bound ^{131}I was determined after four washes. Each value is based on five determinations, using three different IgG preparations: ○, IgG lot No. 183; ●, IgG lot No. 55; △, IgG lot No. 513.

quantity of added IgG for concentration up to 1 mg IgG, but beyond this concentration the amount of additional IgG fixed was not maintained and appeared to approach an asymptotic value in the range of 2·6 mg of added IgG. At 160 μg added IgG, 2·14 per cent of the IgG was bound with an average of $1{\cdot}29 \times 10^5$ molecules per red cell; whereas, at 2600 μg added IgG there was 0·29 per cent of the total IgG bound with an average of $3{\cdot}43 \times 10^5$ molecules/cell.

Table 1 shows the results obtained at low concentrations of IgG. In the range of 1 μg of IgG there were only 50–60 IgG molecules bound per red cell.

ELUTION OF RED CELL BOUND [^{131}I]IgG DURING WASHING

The elution of red cell bound [^{131}I]IgG during the washing procedure was evaluated by following after fifteen successive washes the cell bound ^{131}I and the ^{131}I recovered in the washes. When 1 ml of a 1 per cent red cell suspension was incubated with 200 μg

[^{131}I]IgG, the radioactivity in the washes decreased exponentially with the first four to five washes and, thereafter, remained relatively constant being approximately 1–2 per cent of the cell bound ^{131}I.

A similar pattern of elution with washing was observed when the quantity of [^{131}I]IgG incubated with the cells was decreased. When 100 μg IgG were used the ^{131}I recovered in the washes levelled off to about 0·7 per cent of the cell bound ^{131}I after three washes.

TABLE 1

RED CELL UPTAKE OF [^{131}I]IgG AT LOW CONCENTRATIONS OF IgG

IgG added (μg)	IgG bound × 10^{-3} (μg)	IgG bound (per cent)	Average No. of molecules bound per cell
16·5	93·0 ± 24·0	0·56	3500
1·65	1·46 ± 0·28	0·09	55
0·89	1·33 ± 0·58	0·15	50
0·17	0·39 ± 0·06	0·23	15

0·1 ml of a 10 per cent red cell suspension (10^8), group O, Rh_0(D) positive, was incubated with the [^{131}I]IgG in phosphate buffered saline, pH 7·3, 0·15 M in a total reaction volume of 1·0 ml. The quantity of cell bound ^{131}I was determined after four washes and the values shown represent the mean of five determinations and the standard error of the mean.

EFFECT OF pH AND IONIC STRENGTH ON THE FIXATION OF IgG TO RED CELLS

The quantity of IgG bound to the red cells was affected by pH. Maximum uptake occurred at pH 6·8, at which pH there was 1·8 times more IgG bound to the red cells than at pH 6·0 and 1·6 times more than at pH 7·3. These differences were significant ($P<0·01$).

The effect of ionic strength on the red cell uptake of IgG was studied by adding 0·29 M sucrose to the reaction mixture to reduce the ionic strength. There was a two-fold increase in the amount of IgG fixed to the red cells when the ionic strength was reduced from 0·15 to 0·02.

RELATIONSHIP BETWEEN QUANTITY OF CELL BOUND IgG AND RED CELL CONCENTRATION

Table 2 presents the data obtained when decreasing concentrations of red cells are

TABLE 2

EFFECT OF RED CELL CONCENTRATION ON THE UPTAKE OF [^{131}I]IgG

RBC final concentration (volumes per cent)	IgG bound (per cent)	IgG bound (μg)	Average No. of IgG molecules per cell × 10^5
45	0·48	1·9 ± 0·12	0·016
10	0·96	3·8 ± 0·06	0·14
1·0	0·99	3·9 ± 0·16	1·40
0·10	1·40	5·7 ± 0·14	24
0·05	1·40	5·5 ± 0·50	41
0·02	1·60	6·7 ± 0·23	105

Group O, Rh_0(D) positive red cells in the concentrations indicated were incubated with 390 μg IgG in phosphate buffered saline, pH 6·8, 0·15 M in a total volume of 1·0 ml. The quantity of ^{131}I bound was determined after washing the cells six times. The values represent the mean of five determinations and the standard error of the mean.

incubated with a constant quantity of [^{131}I]IgG. All tests were adjusted to a final volume of 1 ml. As the red cell concentration was decreased, both the relative and absolute quantity of [^{131}I]IgG fixed to the cells increased. The test in which an 0·02 per cent red cell suspension was used had an almost 1000-fold increase in the number of cell bound IgG molecules over the test in which a 45 per cent red cell suspension was used. In spite of the great reduction in the number of red cells the 0·02 per cent suspension bound almost three times the quantity of IgG as was bound by the 45 per cent red cell suspension.

FIXATION OF [^{131}I]IgG, Fab AND Fc ON RED CELLS

Table 3 shows the results obtained after incubating red cells with labelled IgG preparations and papain fragments. There was no difference in red cell uptake of the freshly

TABLE 3

UPTAKE OF [^{131}I]IgG, Fab AND Fc BY RED CELLS

Preparation	μg added to red cells	Added material bound to cells (μg)	Added material bound (per cent)	Average No. of molecules bound per cell × 10^5
IgG	853	5·22 ± 0·13	0·61	2·20
	240	2·63 ± 0·08	1·08	1·05
Fc	290	1·63 ± 0·05	0·57	2·00
	80	0·80 ± 0·02	1·00	0·96
Fab	290	1·85 ± 0·04	0·64	2·30
	80	0·86 ± 0·05	1·08	1·04
IgG (SRC)	896	10·7 ± 0·11	1·19	4·29
	240	3·20 ± 0·20	1·33	1·28

0·1 ml of a 10 per cent red cell suspension (10^8), group O, Rh_0(D) positive, was incubated with the preparations indicated above at pH 7·3, 0·15 M in a total volume of 1 ml. The cells were washed four times after reaction. Values shown represent the mean of five determinations and the standard error of the mean.

prepared IgG and the papain fragments when the results were expressed as average number of molecules bound per red cell. Since the Fab and Fc fragments were prepared from a Cohn Fraction II IgG, the red cell uptake of the undegraded, labelled IgG was also tested. Red cells incubated with Cohn Fraction II IgG (SRC) took up twice as much IgG as they did from the IgG prepared by ultracentrifugation and DEAE chromatography (Table 3).

INFLUENCE OF RED CELL FACTORS ON THE UPTAKE OF IgG

The quantity of IgG bound to the red cells of eight unrelated persons of different ABO groups showed a two-fold variation and ranged from 5·1–10·6 × 10^5 molecules of IgG bound per cell. Under these conditions of testing there was no apparent relationship between ABO group and 'non-specific' uptake of IgG.

No significant difference in uptake of IgG could be demonstrated between 'young' and 'old' red cell fractions obtained by ultracentrifugation. The red cell fraction containing 'old' red cells took up an average of 7·07 × 10^5 IgG molecules per cell and the 'young' red cell fraction bound 7·85 × 10^5 IgG molecules per cell.

UPTAKE OF ^{131}I BOVINE SERUM ALBUMIN (BSA) BY RED CELLS

The uptake of [^{131}I]BSA by red cells was determined following incubation of the cells with different quantities of BSA. As the quantity of BSA was increased from 0·11 to 2·65 mg, there was a progressive increase in the quantity of BSA bound to the cells. When 110 μg BSA was used, there was an average of $1{\cdot}15 \times 10^5$ molecules of BSA bound per cell, which increased to $2{\cdot}69 \times 10^5$ when 2650 μg BSA was used.

EFFECT OF UNLABELLED IgG AND BSA ON THE RED CELL UPTAKE OF [^{131}I]IgG

The effect of unlabelled IgG and BSA was dependent on the quantity of [^{131}I]IgG used for the reaction. When 60 μg of [^{131}I]IgG was used with 0·1 ml of a 10 per cent suspension, there was little effect by unlabelled IgG and BSA on the red cell uptake of [^{131}I]IgG. With 60 μg unlabelled IgG the cells took up 93 per cent of the saline control and when 5000 μg unlabelled IgG was used, the cells took up 86 per cent of the control value. Unlabelled BSA also failed to affect significantly the binding of [^{131}I]IgG when 60 μg of [^{131}I]IgG was used.

When 1280 μg of [^{131}I]IgG was used in the test system, both unlabelled IgG and BSA produced an inhibition of the red cell uptake of [^{131}I]IgG. With high concentrations of unlabelled IgG and BSA there was a 50 per cent reduction of the uptake of [^{131}I]IgG. On a molar basis the effect of BSA was almost twice as great as that observed with unlabelled IgG.

REACTIVITY OF THE RED CELL BOUND [^{131}I]IgG IN THE ANTI-GLOBULIN REACTION

In all experiments the red cells coated with [^{131}I]IgG or [^{131}I]Fab and Fc fragments (Table 3) failed to agglutinate with varying dilutions of a rabbit anti-globulin, a sheep anti-IgG or a rabbit anti-Fab.

DISCUSSION

The results obtained in this study show that human red cells following incubation with [^{131}I]IgG will take up from 0·10 to 3·0 per cent of the applied ^{131}I. The interpretation of these data is subject to a large experimental error because the parameter being measured, the red cell uptake of ^{131}I, represents less than 5 per cent of the ^{131}I in the test system. In addition, because of this low uptake, serious consideration should be given to the possibility that the ^{131}I bound to the red cells is not IgG but a contaminant in the IgG preparation.

^{131}I-labelled substances in the IgG preparations which may bind to red cells include: ^{131}I-labelled fatty acids of the plasma lipids, which could exchange with the red cell membrane lipids (Hughes-Jones and Gardner, 1962), free ^{131}I inorganic iodide which could exchange with the intracellular red cell anions (Hughes-Jones and Gardner, 1962; Costea *et al.*, 1962) and [^{131}I]transferrin which could be bound to reticulocytes and young red cells in the red cell suspension used for testing (Jandl and Katz, 1963; Morgan and Laurell, 1963). Presumptive evidence that the red cell uptake of ^{131}I was not due to a contaminant was obtained from the observation that the uptake of ^{131}I was not proportional to the amount of IgG added to the red cells as the concentration of IgG was increased (Fig. 1).

Direct evidence to show that the red cell uptake of ^{131}I was not due to a contaminant was obtained by testing the IgG preparations for these substances. No ^{131}I-labelled lipoproteins could be detected in the [^{131}I]IgG by use of an exchange procedure in which the [^{131}I]IgG was equilibrated with a large excess of unlabelled high density lipoproteins, low density lipoproteins or with fresh serum. No α_1-, α_2- or β-lipoproteins could be found in the [^{131}I]IgG preparations with the double immunodiffusion technique. By both these criteria, exchange and immunodiffusion, the IgG preparations used in these studies did not contain more than 0·2 per cent [^{131}I]lipids. The red cell uptake of [^{131}I]lipids found by Hughes-Jones and Gardner (1962) was due to the fact that they iodinated whole serum which contains significant quantities of lipids with unsaturated fatty acids.

No data were obtained to indicate that the ^{131}I bound to the red cells was iodide-131. Less than 0·5 per cent of the total ^{131}I in the IgG preparations was in the protein-free form. In addition, no significant increase in ^{131}I red cell uptake was observed when the cells were incubated with an undialysed IgG preparation which contained almost twice as much total ^{131}I as the dialysed IgG. By double immunodiffusion the [^{131}I]IgG preparations were free of [^{131}I]transferrin.

Further evidence that the ^{131}I bound to the red cells was IgG was that the ^{131}I in the IgG preparations displayed the same pattern as IgG in immunoelectrophoresis, double immunodiffusion and DEAE column chromatography. Since the sensitivity of the techniques used to detect contaminants was sufficient to detect concentrations greater than 0·2 per cent, it is evident that the ^{131}I red cell uptake greater than 0·2 per cent was due to IgG or some form of IgG.

Both the cell concentration and the quantity of IgG present during the reaction affect the red cell uptake of IgG. As the IgG added was progressively increased while the cell concentration was maintained constant, there was a progressive increase in the quantity of IgG bound which seemed to approach an asymptotic value. This observation suggests that the binding was limited by the quantity of red cells or the red cell surface area available during the reaction.

When the IgG concentration was maintained at a constant level and the red cell concentration was progressively decreased, there was a progressive increase in both the relative and absolute quantity of IgG bound. With the IgG and cell concentrations used in the present study the red cell uptake of IgG was inversely proportional to red cell concentration. It would appear that, at high red cell concentrations, there may be cell to cell interaction resulting in inhibition of cell uptake of IgG. The data do not offer any clues as to what this mechanism may be.

It is of interest to compare the results of the present study with the reports of earlier investigators. Unfortunately, precise comparisons are not always possible because the reports in the literature do not give the protocols for characterizing the homogeneity of the IgG preparation used by the investigator. Pirofsky *et al.* (1962), using a 10 per cent red cell suspension and 1000 μg IgG, found an average uptake of 2×10^5 IgG molecules per red cell and Costea *et al.* (1962) under the same conditions obtained a value in good agreement, $2{\cdot}36 \times 10^5$ IgG molecules per red cell. In the present study, at the same IgG and red cell concentration, only $0{\cdot}14 \times 10^5$ IgG molecules were bound per red cell (Table 2) which represents only 7 per cent of the uptake observed by these earlier investigators. Hughes-Jones and Gardner (1962), using a 50 per cent red cell suspension, found an average of 5000 IgG molecules per red cell as compared to 1600 IgG molecules in the present study, using a 45 per cent red cell suspension. In all cases in which a comparison was possible,

the results obtained in the present study are consistently lower than those reported previously. The higher red cell uptake of ^{131}I is probably due to protein contaminants other than IgG and to the increased content of aggregated or altered IgG in the preparations used by the earlier investigators (Frommel, Grob, Masouredis and Isliker, 1967).

Many of the conditions which affect antibody binding to red cells have a similar effect on the 'non-specific', non-antibody red cell uptake of IgG. The uptake of [^{131}I]anti-$Rh_0(D)$ is pH dependent with an optimum between pH 6·5 and 7·0 (Masouredis, 1959). Non-antibody binding of IgG by red cells has a similar pH optimum. The red cell uptake of non-antibody IgG can be increased by lowering ionic strength. A similar effect can be shown for the red cell uptake of anti-$Rh_0(D)$ (Atchley, Bhagavan and Masouredis, 1964; Hughes-Jones, Gardner and Telford, 1964).

A number of significant differences between the red cell uptake of anti-$Rh_0(D)$ and the red cell binding of 'non-specific' IgG should be noted. The red cell uptake of antibody is relatively independent of red cell concentration. As the cell concentration is increased the quantity of antibody bound is progressively increased. With many labelled anti-$Rh_0(D)$ preparations, a 30–50 per cent red cell suspension will bind up to 80 per cent of the added radioactivity (Fong and Masouredis, 1967). With 'non-specific' IgG the reverse is the case (Table 2).

The most striking difference between antibody and non-antibody binding of IgG concerns the quantity of IgG bound per cell, 10^5 molecules per red cell for non-antibody fixation and 10^3–10^4 for antibody fixation. This discrepancy in the quantity of IgG bound between the two types of fixation is due to differences in the amount of IgG used for each type of reaction. From 60 to 100 μg of IgG are needed to achieve a binding of 10^5 molecules/cell, whereas less than 1 μg of anti-$Rh_0(D)$ is sufficient to sensitize $Rh_0(D)$ positive cells with 10^4 anti-$Rh_0(D)$ molecules per cell. When comparable quantities of IgG are used for fixation, 1 μg of IgG results in an uptake of about 60 molecules/cell (Table 1).

The effect of albumin on the two types of IgG red cell fixation also serves to distinguish between antibody and non-antibody uptake of IgG. At large concentrations of IgG albumin inhibits the red cell uptake of IgG, whereas albumin appears to increase the uptake of anti-$Rh_0(D)$ (Masouredis, 1966 unpublished observations). It would appear that in the case of non-antibody IgG fixation, albumin inhibits IgG uptake by competing for red cell surface sites. Albumin enhancement of anti-$Rh_0(D)$ uptake is probably associated with the dielectric activity of albumin and the effect of this activity on lowering the zeta-potential as discussed by Polack, Hager, Relckel, Toren and Singher (1965).

The inability of non-antibody red cell bound IgG to react in the anti-globulin reaction is well known. It was first recognized by Boursnell *et al.* (1953) and further studied by Pirofsky *et al.* (1962). Even though the anti-globulin technique is capable of agglutinating red cells sensitized with less than 100 anti-$Rh_0(D)$ IgG molecules (Dupuy, Elliot and Masouredis, 1964), red cells coated with more than 10^5 molecules of non-antibody bound IgG fail to agglutinate in the anti-globulin reaction. This anomalous behaviour of cell bound ^{131}I in the anti-globulin reaction in the present study was not due to cell fixation of non-IgG contaminants in the [^{131}I]IgG preparation. The results of this study do not support the interpretation that the difference between antibody and non-antibody binding is associated with the orientation of the cell bound molecule. No significant difference in the quantity of IgG, Fc or Fab bound non-specifically to red cells was found in the present study (Table 3).

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