3. It is suggested that the effect is due to three factors. (*a*) If magnesia mixture is added to the reduced phosphomolybdate complex and the resulting precipitate analysed colorimetrically, recoveries of inorganic phosphate are low. (*b*) Free molybdic acid is reduced, giving molybdenum blue. Contrary to prevailing opinions, molybdenum blue is extracted from acid solutions by *iso*butanol. (*c*) The specific extinction coefficient is about 4–5 times higher when the colour of the reduced phosphomolybdate complex is developed at 100° than when it is developed at room temperature. This is true for most reducing agents except stannous chloride, with which a higher extinction is obtained at room temperature.

4. The effect is not observed under the milder experimental conditions of Weil-Malherbe & Green (1952). If it had occurred, a stannous chloride method of phosphate estimation, such as was used by these authors, would have been less subject to error than a method using aminonaphtholsulphonic acid.

The author is indebted to Mr R. H. Green for much technical assistance.

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Studies with Marked Antisera

QUANTITATIVE STUDIES WITH ANTISERA MARKED WITH IODINE ^{131}ISOTOPE AND THEIR CORRESPONDING RED-CELL ANTIGENS

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By now a considerable number of workers has begun to use the radioisotope technique in investigations on serological and immunological phenomena (see review by Francis, Mulligan & Wormall, 1951). These methods enable one to trace quantitatively suitably marked antibodies and antigens in amounts beyond the range of any other known methods. The object of the present investigations was the study of the combination of specific serum antibodies with antigens on the red-cell surface by quantitative isotopic methods. Such a study entails marking the antibody molecules in an antiserum with an isotope and allowing the marked antiserum to react with a suspension of red blood cells. The cells are then washed free of uncombined protein and the radioactivity of the washed, sensitized cell suspension measured.

Because of the necessity of washing the sensitized cell suspensions free of all uncombined serum proteins, antigen-antibody systems had to be chosen which do not produce strong agglutination or clumping of the cells, since it would not be possible to wash the aggregates free of all protein other than that specifically bound to the cell, and haemolysis would probably occur if an attempt were made to break them up by mechanical agitation. The following red-cell antigen-antibody systems are suitable. (1) The so-called incomplete Rh antibodies (Race, 1944; Wiener, 1944) combine with the red-cell antigens but for some reason, as yet unknown, fail to bring about agglutination. We have used the incomplete anti-D Rh antiserum with Rh-positive human red cells. (2) The red cells of certain oxen, owing either to the disposition of the antigens on the cell surface, or to some other physical property of their surface, remain unagglutinated when sensitized by potentially agglutinating antibodies. We have used bovine red cells of the 'in-

agglutinable' type (Gleeson-White, Heard, Mynors & Coombs, 1950) with either rabbit anti-bovine red-cell serum or human serum containing the Paul-Bunnell antibody which also has a specific affinity for an antigen on bovine red cells.

The isotope used to mark the antibodies in the sera was elementary radioactive iodine (^{131}I; half-life 8·0 days). The use of iodine as a tracer has many distinct advantages. The iodine isotope is easy to measure, has a conveniently long half-life for *in vitro* studies, and is readily obtainable. If radioactive iodine, in the presence of a suitable quantity of 'carrier' iodine, is allowed to react with protein at physiological pH and at room temperature, in such a way that about one atom is introduced per protein molecule, the iodine appears to be very firmly bound. Moreover, the serological titre of the antibody is not apparently affected by this treatment if allowance is made for the dilution of the antiserum which occurs during iodination.

Early in the experimental work with these serological systems a serious obstacle was encountered in the large adsorption by red cells of marked serum proteins which could not be detected serologically and which appeared to be entirely non-specific. However, once this difficulty was overcome and assurance was gained that the methods were reliable, the investigations were continued along the two following lines: (*a*) measurement of the total specific antibody content of an antiserum in terms of μg. protein; (*b*) determination of the total number of sites of a particular antigen on a red-cell surface.

The quantitative results offer an explanation for certain empirical findings associated with the serological systems studied.

METHODS AND MATERIALS

Preparation and estimation of radioactive materials

Iodination of proteins. A study was made of the best methods available to liberate iodine quantitatively from the iodide, the form in which ^{131}I is normally transmitted from the Atomic Energy Research Establishment. It was felt that, if possible, the resulting iodine solution should contain nothing that could be considered deleterious to the protein. For this reason attention was concentrated on the reaction $KIO_3 + 5KI + 6HCl = 3I_2 + 3H_2O + 6KCl$ (cf. Francis *et al.* 1951). As normally used, in this reaction both the KI and the HCl are present in considerable excess, and the reaction proceeds to completion. However, it is desirable to avoid a large excess of HCl in the subsequent reaction with the antiserum and the need to conserve radioactive iodine means that the KI must not be present in large excess either. It was found that iodine was still liberated quantitatively from the KI with a slight excess of KIO_3 and HCl according to the above equation. Further investigation of this reaction in which an attempt was made by the addition of a buffer to the system after the liberation of the iodine, to reduce the pH to more nearly physiological conditions, revealed the fact that apparently the reaction does not give a quantitative liberation of iodine under these conditions. However, the loss entailed was not serious, and as a standard procedure the solutions were added in the following order to a stoppered test tube:

(*a*) 0·11 ml. KIO_3 (0·107 g./100 ml.).

(*b*) 0·1 ml. KI (0·415 g./100 ml.) to which had previously been added the $Na^{131}I$ solution (usually in small volume).

(*c*) 4 drops 0·1 N-HCl

(*d*) 2 drops 0·2 M phosphate buffer (pH 8·0).

(*e*) The serum to be iodinated (usually between 0·5 and 2·0 ml.).

The serum was always added as quickly as possible after the buffer and the reaction was allowed to proceed for 45 to 90 min. Any trace of iodine remaining was removed by the addition of a few drops of 0·1 N-$Na_2S_2O_4$.

The resulting solution was dialysed in cellophan tubing against 500 ml. quantities of saline (0·9 % (w/v) NaCl) in the cold (1–4°) until the dialysing saline was free from radioactivity. Five changes of saline at intervals of 5 hr. or more sufficed.

It follows that in the iodination procedure and dialysis a certain dilution of the serum protein is incurred. The concentration of the ^{131}iodinated protein was measured by means of Kjeldahl total-N determinations; in serial dilutions, this is designated 'neat'.

Observations upon the stability of the iodine-protein linkage. An attempt was made to determine the stability of the iodine attached to the serum proteins used in these experiments. A quantity of ^{131}I-marked rabbit anti-bovine red-cell serum, after dialysis as described above, was placed at the bottom of a U-shaped fold of new cellophan tubing to avoid any leakage through the tied ends of the sac. The contents of the sac were dialysed against 10 ml. quantities of saline both at 4° and at room temperature for a fortnight. During this time only a very small proportion (2·6 %) of the total radioactivity (allowing for decay) escaped into the saline dialysing medium. It seemed likely that this could be accounted for by a slight reduction in the total quantity of protein present in the sac, due either to a very slow leakage of protein or possibly to bacterial action.

It was found that there was no fixation by red blood cells of quantities of iodide or iodine very much greater than was ever likely to be liberated by the rupturing of the iodine-protein linkage.

The relative combination of iodine with serum and γ-globulin. A study was made of the ^{131}I/total-N ratios in marked whole human serum and in γ-globulin separated therefrom. Preliminary experiments had shown that the ratio for the separated γ-globulin was somewhat less than that for the whole serum (cf. Pressman & Sternberger, 1950). By iodinating with different quantities of added carrier iodine, following in general the method described above, samples of marked serum were obtained with iodine contents varying from about 0·07 to 2 atoms/molecule of protein (assumed average mol.wt. of 1×10^5).

The γ-globulin was separated by using 18 and 12 % (w/v) Na_2SO_4 according to the method of Kekwick (1940). Samples of the initial ^{131}I-marked sera and of the γ-globulin were taken for radioactivity and total-N determinations. From these figures the specific activity in terms of counts/min./μg. protein could be calculated. In order to make possible the calculation of the atoms of iodine/molecule of protein, it was necessary to know the specific activity (counts/min./μg. iodine) of the liberated iodine in each case. This was facilitated by making up the original ^{131}I sample

to a known volume. Portions of this were used for each iodination, and from this also radioactivity standards were prepared. From these figures, accepting an average mol.wt. of 1×10^5 for serum proteins, the atoms of iodine/protein molecule can readily be calculated.

The graph (Fig. 1) shows that the ratio (I/total N in γ-globulin to I)/(total N in whole serum proteins) varies with the average number of atoms of iodine on the serum protein molecules, and passes through a maximum at about one atom of iodine/molecule.

A subsidiary experiment in which separated γ-globulin was iodinated and then reprecipitated with 18% Na_2SO_4 showed that this treatment did not decrease the iodine content of the protein. Moreover, the warming of the precipitating mixture to 35°, which is necessary with Na_2SO_4 precipitation, does not appear to affect the iodine-protein link. This is important because in all the experiments incubation for sensitization was carried out at 37°.

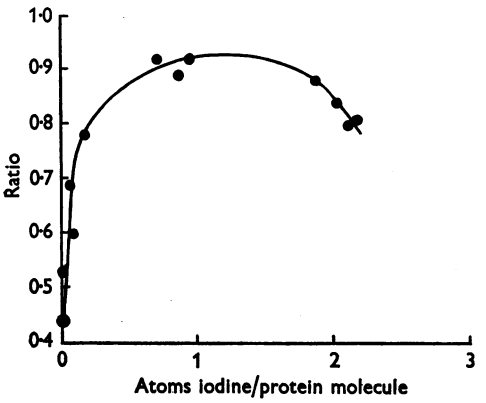

Fig. 1. Curve showing relationship between the average number of atoms of iodine per protein molecule (assumed mol.wt. of serum proteins, 1×10^5) and the ratio expressed as I/total N in γ-globulin to I/total N in whole serum proteins.

Pressman & Sternberger (1950), using precipitates of egg albumin and ^{131}I-marked rabbit anti-egg albumin serum, have shown that the iodine uptake by the antibody was less than that of the total globulin fraction of the serum. There appears to be a discrepancy between the observations in the literature relating to the relative rate of iodination of various serum components. The above authors found that with six or more iodine atoms introduced, on the average, into rabbit antisera, the rate of iodination of the specifically precipitable antibody was less than that of the globulin which was again less than that of whole serum. The presence of antibody was still detectable under these conditions. Masouredis, Melcher & Koblick (1951), on the other hand, using 1·3 iodine atoms in globulin prepared from rabbit antisera without any apparent destruction of antibody, find that the specifically precipitable antibody has an identical iodine content, assuming a mol.wt. of $1{\cdot}6 \times 10^5$ for both. Cohen (1951) has found that iodination of equine antitoxic serum gives a globulin whose rate of iodination is 90–94% of that of the average serum proteins. Pressman & Sternberger (1950) also show that in iodination of mixed rabbit antisera to ovalbumin and to bovine serum albumin the relative rates of iodination of the specifically precipitable antibodies are identical.

Unfortunately, the methods that these authors used to determine the relative rate of iodination of antibody cannot be applied to cellular antigen systems, and therefore, until evidence is available on the point in the particular systems studied here, the γ-globulin-I:serum-I ratio has been taken to apply (in series E) to the antibody fraction also. It is realized that the application of this ratio may not give absolute values.

Radioactivity determinations

Mounting the samples. Radioactivity standards were made as follows: one small drop of the marked protein solution was weighed out and diluted (by weighing) 20–30 times with a 1/10 solution in saline of normal bovine serum. It was found that, by using this 1/10 serum as a diluent, much better reproducibility of the standards was obtained than when normal saline was used alone. After thorough mixing, drops of the diluted ^{131}iodinated protein were weighed out on to concave nickel planchets (General Electric Company, Magnet House, Kingsway, London, W.C. 2) which had previously been thinly lacquered with a pale-gold lacquer. One drop of a 3% solution of polyvinyl alcohol in water was then added and the planchet allowed to dry on a warm hot-plate. The sandwiching of the radioactive material between the lacquer and the polyvinyl alcohol, both of which appeared to be necessary, completely eliminated the tendency of the dried material to flake or curl. It was also found that, by this treatment, the absorption of the β- and γ-radiation from the ^{131}I was only slight, and was in any case sensibly constant for all the samples and standards.

Counting methods and expression of results. The radioactivities of the dried preparations on the planchets were determined by means of conventional, end-window, commercial Geiger-Müller counters and electronic scalers. Two and sometimes three of these sets were used in the radioactivity assays in any one experiment.

The standard samples, five in number for each serum, were usually counted at a definite time in the course of the day, and from these an average figure for the counts/min./μg. ^{131}iodinated protein was obtained. The ratio (representing μg. ^{131}iodinated serum protein on the planchet) of the cell sample count to this average figure was obtained at least twice and on different counting sets, if possible on different days. In a considerable proportion of the cases two determinations of the ratio sufficed. Occasionally more than three determinations were required and this could often be explained by the unreliability of the counts during the first hour after switching on the counting sets. Usually the maximum activity of any of the planchets was between 100 and 500 counts/min., and the minimum activity was 10 or less. It is difficult to work out the overall error arising from such variables as cell sampling and small unavoidable losses in the transference of cells, but the counting errors are certainly less than 5% in almost all cases.

Determination of the error in counting due to self-absorption. Experimental determination of the magnitude of this effect showed that, with about 360×10^6 cells (equivalent to 0·5 ml. 4% cells) on a planchet, the self-absorption was less than 3%. With larger quantities of cells the self-absorption became rapidly appreciable, and to avoid the application of a correction factor, in these cases the cell suspension was divided and mounted on two planchets.

Serological materials and methods

Red cells. Bovine and human red blood cells have been used in these experiments. Defibrinated blood was obtained twice weekly from cow 5157. The cells of this animal belonged to the 'inagglutinable' class described by Gleeson-White *et al.* (1950). Human blood (Rh-positive and Rh-negative) was taken by venepuncture into Wintrobe's oxalate mixture (Wintrobe, 1942). Some samples were taken by pricking the ear and collecting the blood in 0·6% citrate-saline (6 g. trisodium citrate in 1 l. saline). The only Rh antigen we have employed has been the D antigen, and for this work red cells are referred to as Rh-positive if they possess this antigen, Rh-negative if they do not. The probable antigenic structure of the cells is indicated in each case (e.g. (CDe/cde), cf. Race & Sanger, 1950).

The cells were washed three times in saline before use, and the final suspension of known concentration was made in saline or other diluent as indicated in the individual experiments. The final cell concentrations were checked by counting the cells in a haemocytometer.

Sera. (*a*) *Rabbit anti-bovine red-cell sera.* Serum was obtained from rabbit 1876 before and after one and two courses of injections with washed bovine red cells. Each course consisted of six intravenous injections of 1 ml. 10% cell suspension.

The haemolysin titres were estimated by titrating the heat-inactivated sera in 0·1 ml. amounts and adding 0·1 ml. 1% bovine red-cell suspension and 0·1 ml. (4 minimum haemolytic doses) of guinea pig complement. The degree of haemolysis was read after 30 min. incubation at 37°. The titre of the pre-injection serum was less than 5, while after the first course of injections it was 1280, and 5120 after the second course.

(*b*) *Human anti-bovine red-cell sera.* Human serum containing the Paul-Bunnell antibody reactive against bovine red cells was obtained from a patient (*C*) who had contracted infectious mononucleosis. The haemolysin titre of this serum for bovine red cells was 5120. Normal human sera have been used in control tests.

The sera in (*a*) and (*b*) were heat-treated at 56° for 30 min. before iodination. Certain samples, as indicated in the individual experiments, were absorbed with bovine red cells also before iodination.

(*c*) *Human incomplete anti-D Rh sera.* The sensitization of Rh-positive red cells by non-agglutinating incomplete antibodies in Rh antisera may be demonstrated by the antiglobulin sensitization test (Coombs, Mourant & Race, 1945). In this test the cells, after sensitization, are washed free of unabsorbed protein and then tested for adsorbed antibody globulin by means of a rabbit anti-human globulin serum. Sensitized cells are agglutinated by such an anti-globulin serum.

By this procedure the titre of incomplete antibody (anti-D) was 1024 in serum *W* and 2048 in sera *B* and *Bgl.* (Serum *B* contained a trace of incomplete anti-C (titre 8) also.) These human sera were not heat-inactivated before use, but the testing systems were compatible with regard to the ABO blood grouping systems.

The reactions of anti-Rh sera may be controlled serologically either by using Rh-negative cells or by using normal sera on Rh-positive cells. Neither of these two procedures, however, is without certain limitations as a control for the non-specific adsorption of marked protein. The main limitation is inherent in the fact that the non-specific adsorption appears to vary slightly from serum to serum and also with cell samples from different individuals.

The effect of iodination on the antibody titre. Four samples of heat-inactivated infectious mononucleosis serum (*C*) were iodinated, each with increasing amounts of carrier iodine as described previously. After dialysis all the ^{131}I-marked sera were adjusted to the same protein concentration as was also an un-iodinated dialysed control sample. Each sample was titrated for its haemolysin end point against bovine red cells. Tests were made with both doubling and four-fifth progressive dilutions of the samples.

The samples 1–4 contained 0·03, 0·2, 1·8 and 2·7 iodine atoms/protein molecule respectively. No drop in the serological titre could be detected when compared with the control except in sample 4. Even in this case, the last tube of the 'four-fifth' titration to show any haemolysis was the same as in the control, but the haemolysis was definitely weaker in this tube.

From these results it would seem that an average of 1·8 iodine atoms/serum protein molecule can be introduced without destroying any antibody or interfering with its combining properties. A similar experiment carried out with the incomplete anti-D Rh serum *B* again gave no evidence of any effect on the serological titre of the serum with as much as 1·8 iodine atoms/molecule. These results

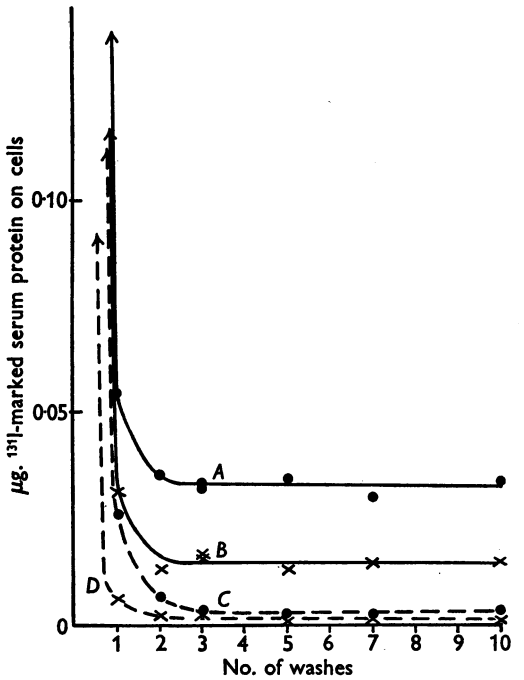

Fig. 2. The effectiveness of washing Rh-positive and -negative human red cells with saline after previous treatment with 0·1 ml. ^{131}I-marked incomplete anti-D serum (^{131}I-*B*, protein 0·11%). Curve *A*, 0·5 ml. 4% suspension (A CDe/CDe) cells; curve *B*, 0·5 ml. 1% (A CDe/CDe) cells; curve *C*, 0·5 ml. 4% (A cde/cde) cells; curve *D*, 0·5 ml. 1% (A cde/cde) cells.

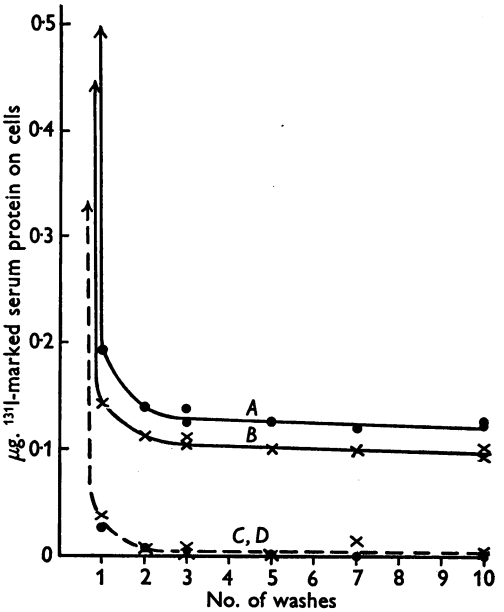

Fig. 3. The effectiveness of washing 'inagglutinable' bovine red cells (0·5 ml. 1% suspension) with saline after previous treatment with 0·1 ml. serum at 37°. ^{131}I-marked human infectious mononucleosis serum (protein 0·26%), incubated for curve *A*, 40 min.; curve *B*, 5 min. ^{131}I-marked normal human serum (protein 0·24%), incubated for curve *C*, 40 min.; curve *D*, 5 min.

are in general agreement with the observations of Masouredis *et al.* (1951) with rabbit antibody and of Cohen (1951) using equine diphtheria antitoxin.

Measurement of the adsorption of ^{131}I-marked antibody on red cells. The following procedures were used in all the experiments, unless otherwise stated.

The marked serum was 'titrated'* with 'doubling dilutions' in a constant volume. To this 'titration' was added equal volumes of a known concentration of red cells. In the early experiments saline was used as the diluent for the serum and red-cell suspensions, but in later experiments this was replaced by undiluted heat-inactivated unmarked serum compatible with the test. In these later tests the red-cell suspensions were prepared 1 hr. before they were added to the 'titrations'. The contents of the tubes were mixed and incubated at 37° for 40 min. The tubes were then centrifuged and the packed cells washed three times with 2 ml. quantities of saline. The final deposit of cells in each tube was resuspended in three drops of saline and transferred to a planchet for radioactivity determinations.

Investigation of the effectiveness of washing treated cells with saline. In these experiments an attempt was made to investigate the relative effectiveness of repeated washing with saline of cells treated with ^{131}I-marked sera.

(*a*) *Using ^{131}I-marked anti-D Rh serum* (^{131}I-B). Eight tubes were set up each containing 0·1 ml. ^{131}I-*B* and 0·5 ml. 1% suspension of Rh-positive cells in unmarked heat-inactivated human serum. A similar series of tubes were set up with Rh-negative cells. The contents of the tubes were incubated, centrifuged and the resulting eight cell deposits of each series were washed 0, 1, 2, 3, 3, 5, 7 and 10 times respectively. Each washing was with 1·6 ml. saline. The whole experiment was repeated using a 4% cell suspension with comparable results as shown in Fig. 2. It will be seen that by three washes all the loosely attached marked protein had been removed and no appreciable further removal took place even with ten washes.

(*b*) *Using ^{131}I-marked infectious mononucleosis serum* (^{131}I-C). A similar experiment was performed with bovine red cells treated with ^{131}I-*C* and with ^{131}I-marked normal human serum. In this case, only one concentration of cells (1%) was used but two different periods for sensitization were employed (5 min. and 40 min.). The findings (Fig. 3) were identical to those of (*a*).

In the experiments described in series D, it was necessary to transfer as quantitatively as possible all the free ^{131}I-marked serum with the least possible increase in bulk. In the two experiments, (*a*) and (*b*) above, determinations show that only 1–2% of the total quantity remained trapped in the cell deposit before washing. In (*a*), washing this deposit with one drop of saline removed 60% or more of this remaining ^{131}I-marked serum. For this reason initial washing with one drop of saline was employed in the experiments in series D.

EXPERIMENTAL

Series A. 'Titrations' of marked antisera against red-cell suspensions in saline

Expt. A 1. *'Titration' against bovine red cells of ^{131}iodinated γ-globulin prepared from rabbit anti-bovine red-cell serum.* The γ-globulins for this experiment were prepared from pre-injection and second course sera from rabbit 1876 by three precipitations at 1·39M concentration of $(NH_4)_2SO_4$, following closely the method of Cohn, McMeekin, Oncley, Newell & Hughes (1940).

The γ-globulins were iodinated separately as described above, and 'titrated' in 0·1 ml. amounts with a 1% suspension of bovine red cell. The haemolysin titre of the γ-globulin preparation of the antiserum, both before and after iodination, measured under comparable conditions, was 512.

The results, together with other relevant data, are shown in Fig. 4. It is of interest to note that radioactivity is still detectable on the cells beyond the serological end point. The slight adsorption from the pre-injection ^{131}iodinated γ-globulin might have been due to a small amount of naturally occurring antibody, but was probably non-specific.

Expt. A 2. *'Titration' of ^{131}I-marked rabbit anti-bovine red-cell serum (1st course) and ^{131}I-marked infectious mononucleosis serum against bovine red cells.* The two antisera and two appropriate normal sera were heat-inactivated, and the two normal sera were then absorbed free of antibodies for bovine red cells. After iodination, the ^{131}I-marked sera were 'titrated' in 0·2 ml. amounts with an equal volume of a 1% suspension of bovine red cells.

* In this work 'titration' is used to indicate that the procedure carried out follows that of a normal serological titration (but without the possibility of an end point).

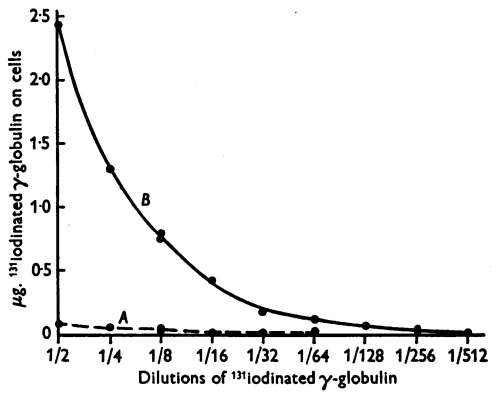

Fig. 4. 'Titration' (curve *A*) of ^{131}I-marked rabbit γ-globulin ('neat', 0·22% protein) from pre-injection serum; (curve *B*) ^{131}I-marked rabbit γ-globulin ('neat', 0·27% protein) from second course anti-bovine red-cell serum against 0·1 ml. 1% suspension of bovine red cells in saline.

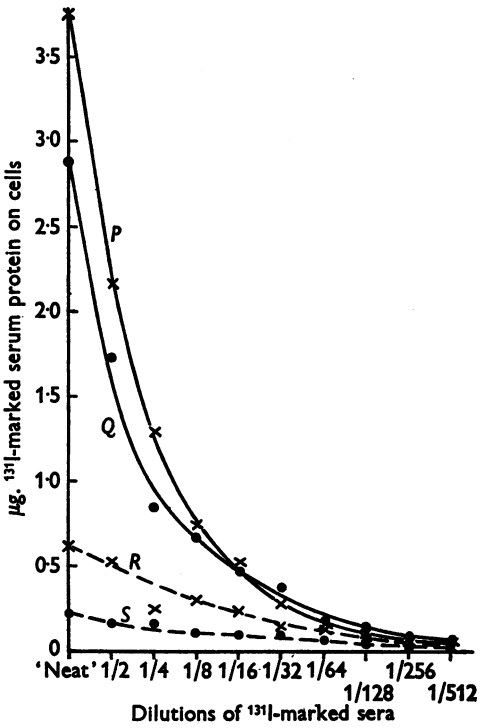

Fig. 5. Comparative 'titrations' of ^{131}I-marked rabbit anti-bovine red-cell serum and ^{131}I-marked human infectious mononucleosis serum (^{131}I-*C*) against 0·2 ml. 1% suspension of bovine red cells in saline. Curve *P*, ^{131}I-*C* ('neat', 2·6% protein); curve *Q*, ^{131}I-marked rabbit anti-bovine red-cell serum ('neat', 2·3% protein); curve *R*, ^{131}I-marked normal human serum ('neat', 2·6% protein); curve *S*, ^{131}I-marked preinjection rabbit serum ('neat,' 2·0% protein).

Fig. 5 shows that the two antisera gave comparable results. The quite considerable amount of marked protein adsorbed from the two normal sera could not be due to specific antibody as these sera had been previously absorbed with bovine red cells.

Expt. A 3. *'Titration' of* ^{131}I-*marked incomplete anti-D Rh serum* ^{131}I-W) *against Rh-positive and -negative human red cells.* In this experiment only one serum was used, ^{131}I-*W*, which showed no reduction of its sensitizing titre of 1024 after iodination. The specificity of the serological reaction was controlled by 'titrating' the serum against Rh-negative cells, as well as against Rh-positive cells. The 'titrations' were carried out using 0·1 ml. amounts of ^{131}I-*W* and 0·1 ml. 1% cell suspensions.

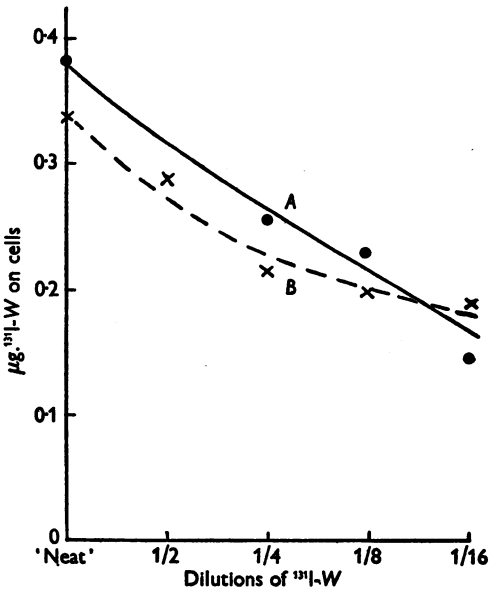

Fig. 6. 'Titrations' of 0·1 ml. ^{131}I-marked incomplete anti-D human serum (^{131}I-*W*, 'neat', 2·3% protein) against 0·1 ml. 1% saline suspensions of Rh-positive and -negative cells. Curve *A*, Rh-positive (O cDE/cde) cells; curve *B*, Rh-negative (O cde/cde) cells.

From Fig. 6 it may be seen that there could be no certainty of differentiating at any dilution of the serum between the Rh-positive and -negative cells by the adsorption of ^{131}iodinated protein. Yet serologically the differentiation was unequivocal, even at a dilution of 1/1024. The explanation of these results, as was made evident by subsequent experiments, is that the specific adsorption on the Rh-positive cells is very small in comparison with the non-specific adsorption.

Observations on the experiments in series A. In the experiments with antigens on bovine red cells the specific adsorption of the ^{131}I-marked antibody was clearly differentiated from the non-specific. In contrast the results with human cells and the in-

complete anti-D serum suggested that the specific antibody adsorption was very small and was in fact practically overshadowed by the non-specific adsorption.

It was obvious from these results that this quantitative study of specific antibody adsorption could not progress until the problem of non-specific adsorption had been overcome. The experiments in series B were devoted to this problem.

Series B. Reduction of the apparently non-specific adsorption of ^{131}I-marked serum proteins on to red cells

A number of attempts have been made to reduce the non-specific adsorption of ^{131}I-marked serum proteins on to red cells.

(*a*) *Effect of temperature.* As this non-specific adsorption by a saline suspension of cells brought to mind serological phenomena described under such terms as 'non-specific cold agglutination' and 'cold autoagglutination' it was decided to study the influence of temperature on the adsorption. A serologically compatible system was chosen for study consisting of ^{131}-iodinated γ-globulin from rabbit serum and a saline suspension of cells from the same animal, and the interaction studied at 16 and 39°. Although the non-specific adsorption was reduced at the higher temperature, it was by no means completely abolished. Adsorption to about the same extent also took place from a ^{131}I-marked rabbit albumin solution, a finding which supported the probability that the adsorbed protein was not specific antibody.

(*b*) *Effect of citrate.* Dacie (1950), in describing an apparently non-specific adsorption of globulin from unheated human serum on to human red cells in the cold, found that the adsorption could be partially inhibited by 0·6% sodium citrate. However, the use of this medium did not prevent the adsorption under discussion here.

(*c*) *Prior adsorption of unmarked protein.* The red cells, after being washed in saline and before use in the experiment, were suspended for 1 hr. at room temperature in antibody-free heat-inactivated unmarked serum, to enable the cells to adsorb non-specifically unmarked serum proteins before coming into contact with the ^{131}I-marked serum. In addition serial dilutions of the ^{131}I-marked sera were carried out with the same unmarked serum instead of with saline. It was hoped that this treatment would decrease the non-specific adsorption of marked protein while still allowing the specific antibody adsorption to proceed unimpaired. The two following experiments demonstrate the use of unmarked serum as diluent for cells and serum to reduce the non-specific adsorption.

Expt. B1. Bovine red cells with ^{131}I-marked infectious mononucleosis serum and ^{131}I-marked normal human serum. The details of this experiment are shown in Fig. 7. The results show that it is possible to reduce the non-specific adsorption to a very low value by using unmarked serologically compatible serum as the diluent. The effect is the same whether bovine or human serum is used. Washing the cells with serum rather than saline did not reduce the non-specific adsorption any further. The reductions due to this treatment were similar in the two sera and the resulting clearly defined difference between the two adsorptions presumably represented specific antibody. Thus the non-specific adsorption had been largely removed while leaving the specific antibody adsorption apparently unchanged.

Expt. B2. Rh-positive and -negative cells with ^{131}I-marked incomplete anti-D Rh serum (^{131}I-B). The cells used in this experiment were Rh-positive and Rh-negative. 1% suspensions were made in saline and also in heat-inactivated normal human serum from an O Rh-negative person. 'Titrations' in 0·1 ml. amounts of ^{131}I-*B* were set up as indicated in Fig. 8.

After the usual incubation, the cells were centrifuged and washed in saline since, as found in expt. B1, washing with unmarked serum does not further reduce the non-specific adsorption. The results of these 'titrations' are shown in

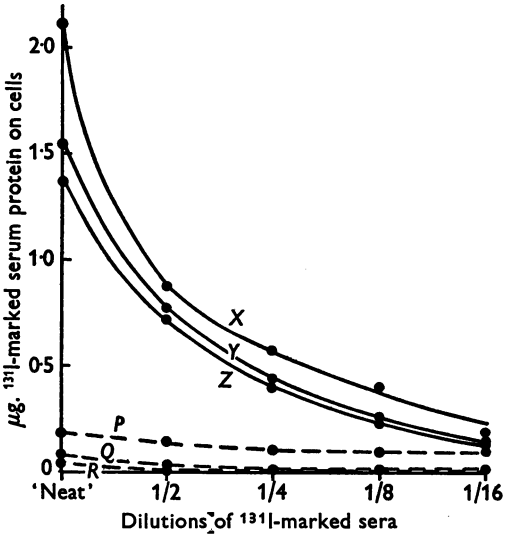

Fig. 7. The use of unmarked serum as diluent for cells and serum to reduce the non-specific adsorption of ^{131}iodinated protein on to bovine red cells. 'Titrations' of 0·1 ml. ^{131}I-marked normal human serum (absorbed with bovine red cells, 'neat', 2·5% protein) against 0·1 ml. 1% bovine cells: diluent for cells and ^{131}I-marked serum, curve *P*, saline; curve *Q*, human serum; curve *R*, bovine serum. Similar 'titrations' of ^{131}I-marked infectious mononucleosis serum ('neat', 3·0% protein): diluent for cells and ^{131}I-marked serum, curve *X*, saline; curve *Y*, human serum; curve *Z*, bovine serum. The cell suspensions were made from a 10% saline suspension by diluting with either (*a*) saline, (*b*) heat-inactivated human AB serum (absorbed with bovine red cells) or (*c*) heat-inactivated bovine serum.

Fig. 8. By the previous incubation of the cells in unmarked serum, the adsorption of ^{131}iodinated protein on to both the positive and negative cells was reduced very considerably, although the difference between the adsorption on positive and negative cells remained unchanged.

Observations on the experiments in series B. The use of unmarked compatible serum as a diluent for both cells and ^{131}I-marked sera apparently did not interfere with antibody adsorption in any way that could be shown serologically or by isotope measurements. At the same time this procedure overcame sufficiently the complication due to the non-specific adsorption to allow quantitative studies on the adsorption of ^{131}I-marked antibody to be pursued. From these and other experiments not reported here it can be stated that the higher the ratio (in terms of total-N concentration) of unmarked serum to ^{131}I-marked anti-D Rh serum the more effective it is in reducing the non-specific adsorption. This treatment is not satisfactory when the ratio is less than 20.

The amount of specific anti-D antibody that was adsorbed on to Rh positive cells appeared to be very small when compared with the amount of ^{131}I-marked antibody that was adsorbed on to bovine red cells from either infectious mononucleosis serum or a rabbit anti-bovine red-cell serum. The smallness of this adsorption seemed puzzling at first as the anti-D serum used had a very high antibody titre, but the results could be interpreted as suggesting that there are relatively few D-receptors on Rh-positive cells. Experiments in the following series support this explanation.

Series C. The effect of the red-cell concentration on antibody adsorption

Expt. C 1. *With ^{131}I-marked infectious mononucleosis serum* (^{131}I-C). Bovine red-cell suspensions were made in heat-inactivated unmarked human serum which had previously been absorbed with bovine cells. This same serum was also used as a diluent for the ^{131}I-marked serum 'titrations'.

Suspensions of 4, 3, 2, 1 and 0·5% cells were made up and 0·5 ml. quantities of each respectively were added to a series of comparable 'titrations' of ^{131}I-*C* in 0·1 ml. amounts. 'Titrations' using the 4 and 0·5% cell suspensions were also set up with ^{131}I-marked normal human serum which had been absorbed with bovine red cells before iodination.

From Fig. 9 it may be seen that increasing the cell concentration from 0·5 to 4% hardly affects the amount of ^{131}I-marked antibody adsorbed. From

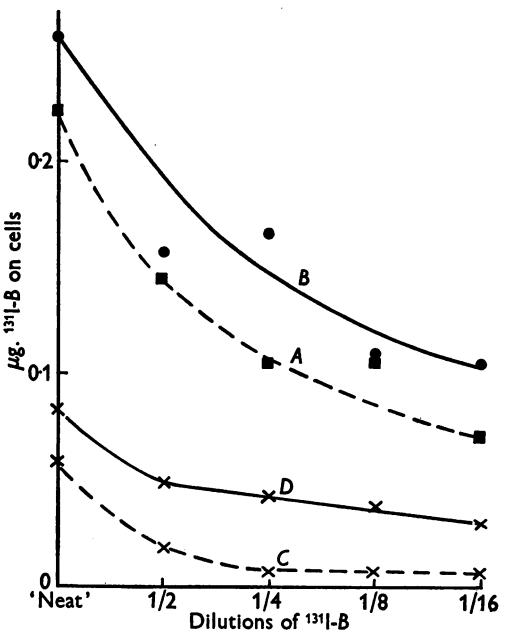

Fig. 8. The use of unmarked serum as diluent for cells and serum to reduce the non-specific adsorption of ^{131}iodinated protein on to human red cells. 'Titrations' of 0·1 ml. ^{131}I-marked incomplete anti-D Rh serum (^{131}I-*B*, 'neat', 2·1% protein) against 0·1 ml. 1% cell suspension. Curves *A* and *C*, Rh-negative cells; curves *B* and *D*, Rh-positive cells. Diluent for cells and ^{131}I-marked serum; curves *A* and *B*, saline; curves *C* and *D*, human serum.

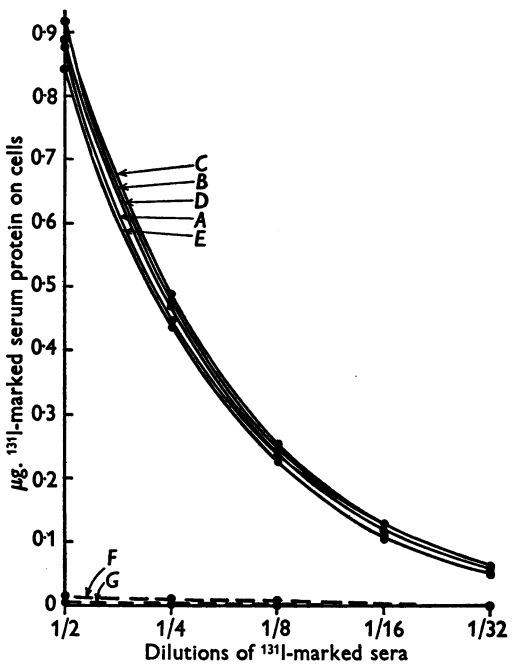

Fig. 9. The effect of the concentration of 'inagglutinable' bovine cells on antibody adsorption. 0·1 ml. ^{131}I-marked infectious mononucleosis serum ('neat', 3·1% protein) with 0·5 ml. cell suspensions in the following concentrations: curve *A*, 4%; curve *B*, 3%; curve *C*, 2%; curve *D*, 1%; curve *E*, 0·5%. Curves *F* and *G*, 0·5 ml. 4% and 0·5% cell suspensions respectively, treated with 0·1 ml. ^{131}I-marked normal human serum ('neat', 2·3% protein).

this it appears that 0·5 ml. of a 0·5 % suspension of bovine cells is capable of absorbing nearly all the antibody present in 0·1 ml. of the 1/2 dilution of the ^{131}I-marked serum sample, a serum whose titre was over 5000. This suggested that the cells have a relatively large number of antigen sites for this antibody.

Expt. C 2. With ^{131}I-marked incomplete anti-D Rh serum (^{131}I-B). In outline this experiment was similar to Expt. C 1, but instead of controlling any non-specific adsorption by using a ^{131}I-marked normal human serum, the control was effected by 'titrating' ^{131}I-*B* against Rh-negative cells, as well as against Rh-positive cells. The various cell suspensions were made up in compatible A, Rh-negative, heat-inactivated human serum which was also used for the serum dilutions. The antiserum was again used in 0·1 ml. amounts and the cell suspensions in volumes of 0·5 ml.

The results (Fig. 10) differ greatly from those of the previous experiment. The measure of ^{131}I-marked antibody adsorbed increased markedly with the increase in cell concentration. The adsorption on the 1 % suspension of Rh-positive cells was almost exactly twice that on the 0·5 % suspension, but the increase in adsorption with higher concentrations became progressively less.

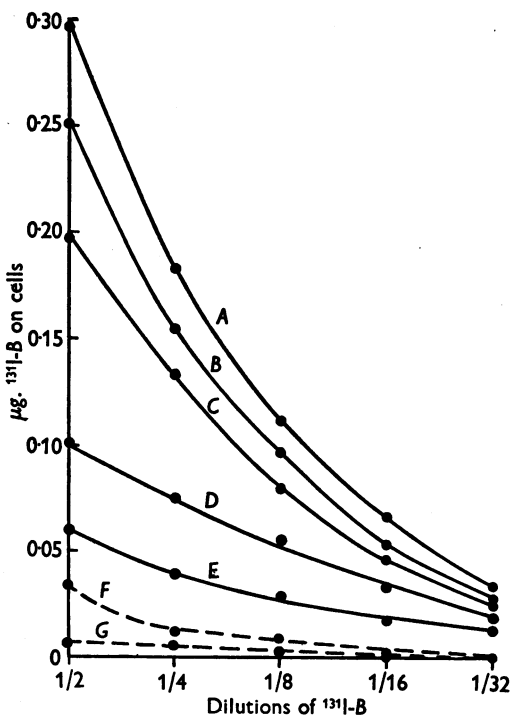

Fig. 10. The effect of the concentration of Rh-positive human cells (O CDe/cDE) on antibody adsorption. 0·1 ml. ^{131}I-marked incomplete anti-D Rh serum (^{131}I-*B*, 'neat', 1·9 % protein) 0·5 ml. cell suspensions in the following concentrations: Rh-positive cells, curve *A*, 4 %; curve *B*, 3 %; curve *C*, 2 %; curve *D*, 1 %; curve *E*, 0·5 %; Rh-negative cells (O cde/cde), curve *F*, 4 %; curve *G*, 0·5 %.

With this system therefore it appeared that the number of antigen sites on the positive cells was the factor limiting antibody adsorption. This is in contrast to the findings in the previous experiment in which the concentration of the antibody in the serum appeared to be the limiting factor.

Series D. Estimation of the specific red-cell antibody in a serum

In the two following experiments a known volume of ^{131}I-marked antiserum was repeatedly absorbed with fresh applications of homologous red cells until no further antibody could be removed. The summation of the specifically combined antibody adsorbed on all the cells, expressed as serum protein, was taken as the measure of the original antibody content of the serum.

It is obviously necessary to be assured that no antibody is destroyed during the iodination process. It was shown (p. 748) that the sera used gave the same serological end point before and after iodination, but it is fully realized that ultimately a better quantitative criterion would be required to prove that no antibody had in fact been destroyed. We have given here a value for the total antibody in terms of serum proteins. These values may require subsequent correction when figures are discovered for the relative iodine uptake of the antibodies compared with serum proteins.

Equal volumes of the cell suspension in unmarked compatible serum were placed in a number of tubes which were centrifuged and the supernatant fluid removed. The volume of the ^{131}I-marked serum, whose antibody content was to be determined, was then added to the cell deposit in the first tube and incubated for 20 min. at 37°. The contents of the tubes were centrifuged and the ^{131}I-marked serum transferred to the next tube in the series. The cell deposit in the first tube was washed with one drop of the unmarked diluent serum (cf. p. 749) and the tube recentrifuged. The supernatant drop was then added to the second tube. This second tube was incubated as before and the procedure repeated for as many times as was necessary to exhaust the serum of specific antibody. Finally all the cell deposits were washed three times with saline as usual before they were transferred to the planchets.

Expt. D 1. Total antibody to bovine red cells in ^{131}I-marked infectious mononucleosis serum (^{131}I-C). 0·5 ml. of a 1/2 dilution of ^{131}I-*C* was repeatedly absorbed with packed cell deposits from 0·5 ml. 0·5 % bovine red-cell suspensions. To control the non-specific adsorption, comparable tests were made with ^{131}I-marked normal human serum (^{131}I-*M*) which had previously been absorbed with bovine red cells. The diluent for cells and the marked sera was inactivated unmarked human serum, also previously absorbed with bovine red cells.

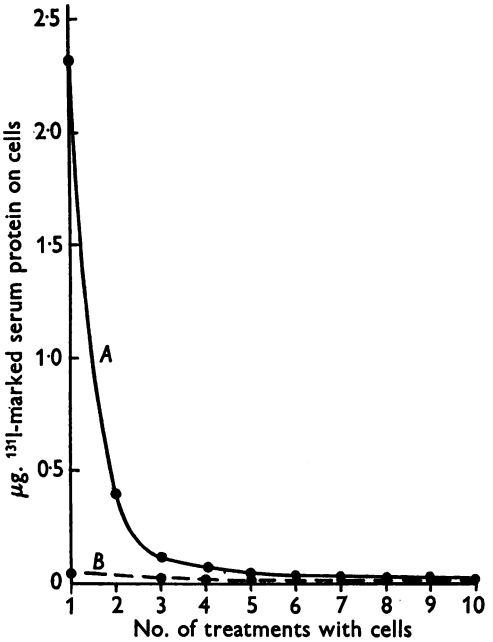

Fig. 11. Assessment of specific red cell antibody content of serum. Curve *A*, absorption of total antibody from 0·5 ml. ^{131}I-marked human infectious mononucleosis serum (protein 1·1%) by repeated treatment with cell deposits from 0·5 ml. 0·5% 'inagglutinable' bovine cell suspension. Curve *B*, identical treatment of 0·5 ml. ^{131}I-marked normal human serum (protein, 1·1%).

The amounts of ^{131}I-marked antibody adsorbed on each sample of cells is shown in Fig. 11. By subtracting the small amount of protein adsorbed from ^{131}I-*M* from that adsorbed from ^{131}I-*C*, a value for the total specific bovine red-cell antibody in the antiserum (in terms of ^{131}I-marked serum protein) could be summated. This amounted to 2·85 μg. in the 0·5 ml. of 1·06% ^{131}iodinated protein which is equivalent to 38 μg. protein per ml. 7% protein. It appears that the concentration of ^{131}I-*C* in the sample absorbed is relatively unimportant, for in another experiment in which 0·18% ^{131}I-*C* was used, a value of 39 μg. protein per ml. 7% protein was obtained.

The antibody in ^{131}I-*C* was almost completely removed by absorption with boiled bovine red cells, and was left unaffected by boiled guinea pig kidney, illustrating its Paul-Bunnell nature.

Expt. D2. Total incomplete antibodies in ^{131}I-marked Rh serum (^{131}I-B). The same procedure was adopted as in the last experiment. 0·5 ml. of a 1/8 dilution of ^{131}I-*B* was repeatedly absorbed with Rh-positive cells using for each absorption deposits from 1·0 ml. 4% cell suspension. The figure for the non-specific adsorption was obtained in a comparable test with Rh-negative cells. The diluent for the cells and marked serum was heat-inactivated serum from an A Rh-negative person.

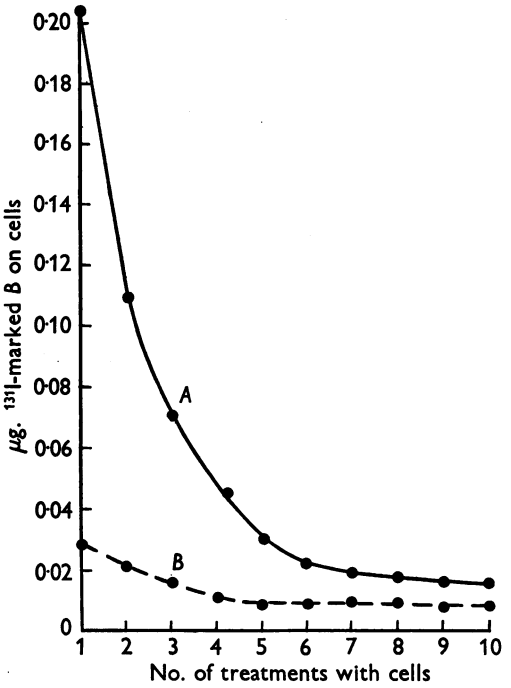

Fig. 12. Assessment of specific red cell antibody content of serum. Curve *A*, absorption of total antibody from 0·5 ml. ^{131}I-marked incomplete anti-D human serum (^{131}I-*B*, protein 0·11%) by repeated treatment with cell deposits from 1·0 ml. 4% suspension of Rh-positive cells (A CDe/CDe). Curve *B*, identical treatment with Rh-negative cells (A cde/cde).

The graph of the results (Fig. 12) is different from that of the previous experiment in that the serum required more absorptions to exhaust it of antibody. In fact, a minute amount of antibody appeared to be left unabsorbed after ten treatments, a fact which could be demonstrated serologically.

The assessment of the total antibody content of the ^{131}I-*B* gave a value of 54 μg./ml. 7% protein (expressed as ^{131}I-marked serum protein).

Series E. Assessment of the number of antigen sites of a particular specificity on the red-cell surface

If the antigen sites of a particular specificity on a known number of cells are saturated with antibody, then the number of antibody molecules adsorbed divided by the number of cells used should give the average number of functional sites on the individual cell provided that certain assumptions are valid. These are: (i) that a single antigen site combines with a single antibody molecule, and (ii) that all available functional sites are saturated when no further antibody is adsorbed. It is possible that this assumption may be invalidated by steric hindrance which would have the effect of apparently reducing the number of functional sites; this effect

would presumably be greater the larger the number of antigen sites per cell. That very little or no antibody is eluted during the washing process is shown by the results of the washing experiments (Figs. 2 and 3).

In performing the experiments in series E, equal amounts of packed-cell deposits were incubated for 20 min. periods a varying number of times with fresh quantities of the ^{131}I-marked serum. After this treatment each sample of cells was washed with saline as usual and finally transferred to the planchets.

Expt. E1. Assessment of the number of Paul-Bunnell antigen sites on a single bovine red cell. 0·5 ml. amounts of a 0·5% suspension of bovine cells in unmarked human serum (inactivated and previously adsorbed with bovine cells) were placed in each of twelve tubes. The tubes were centrifuged, the supernatant fluid removed and the cell deposits used in the experiment. Four of these cell deposits were treated 1, 3, 5 and 9 times respectively as described above, with 0·1 ml. ^{131}I-*C*, four with ^{131}I-*C* which had been adsorbed with boiled ox cells before iodination, and four with ^{131}I-*M*.

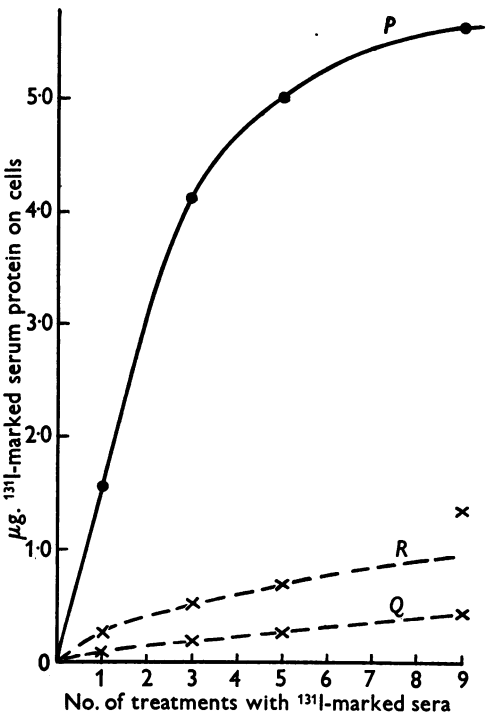

Fig. 13. Assessment of the number of Paul-Bunnell antigen sites on bovine red cells. 0·5 ml. 0·5% 'inagglutinable' bovine cells: curve *P*, repeated treatment with 0·1 ml. quantities of ^{131}I-marked infectious mononucleosis serum (protein, 2·0%); curve *Q*, similar treatment with ^{131}I-marked infectious mononucleosis serum, absorbed with boiled bovine cells before iodination (protein, 1·2%); curve *R*, similar treatment with ^{131}I-marked normal human serum (protein 2·0%).

The graph (Fig. 13) shows the amount of antibody adsorbed after the various treatments. Further experiments, in which the saturation of the cells was assured, gave similar results.

Expt. E2. Assessment of the number of D-sites on a single Rh-positive human red cell. 2% suspensions of Rh-positive and Rh-negative cells in heat-inactivated A_1B Rh-negative serum were prepared. Deposits from 1·0 ml. of these suspensions were repeatedly treated with 0·2 ml. 1/11 dilution of ^{131}I-*Bgl*, 3, 5, 7, 9 and 11 times respectively as previously described. The only antibody in serum *Bgl* reactive against the Rh-positive cells used was anti-D. The results (Fig. 14) show that there is no appreciable increase in antibody adsorption after the seventh treatment with the serum.

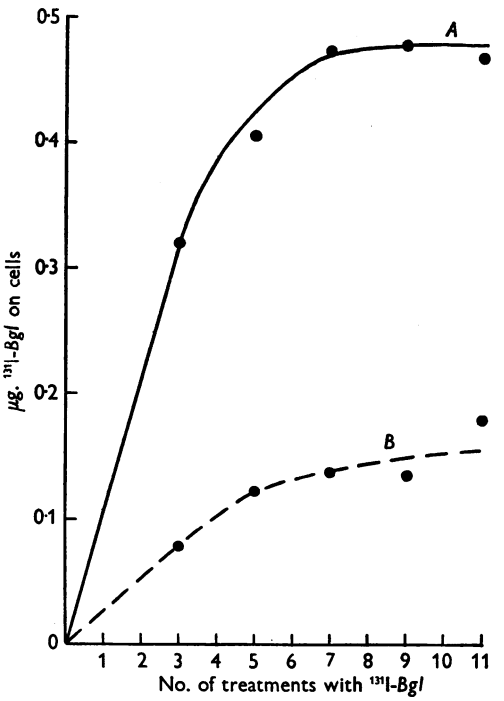

Fig. 14. Assessment of the number of D Rh antigen sites on human red cells. Curve *A*, 1·0 ml. 2% Rh-positive human cells (A_1B CDe/CDe) repeatedly treated with 0·2 ml. quantities of ^{131}I-marked incomplete anti-D human serum (^{131}I-*Bgl*, protein, 0·4%); curve *B*, similar treatment of Rh-negative cells (A_1B cde/cde).

The number of sites on a single red cell can be calculated from the following expression.

$$\frac{P \times N}{\text{Mol.wt.} \times 10^6 \times N \times R'}$$

where P = weight (μg.) of antibody adsorbed at saturation,

N = Avogadro's number ($6{\cdot}02 \times 10^{23}$),

Mol.wt. = molecular weight of antibody,

N = number of cells used,

R = ratio antibody I : serum I.

In calculating from this expression the number of sites on a single cell, at present a figure for the molecular weight of the antibody has to be assumed. Deutsch, Alberty, Gosting & Williams (1947) produce evidence that β-haemagglutinins of human plasma represent a small amount of the high molecular weight portion of the γ_1-globulin. Davis, Moore, Kabat & Harris (1945) show that the Wassermann antibody is associated with both the light and heavy components of whole syphilitic serum ($S = 7$ and 19 respectively). Kabat (1939), in studies on pneumococcal antibodies, states that whereas in cow, horse and pig these have a molecular weight of $9{\cdot}9 \times 10^5$, in human, rabbit and monkey the figure is $1{\cdot}6$–$1{\cdot}8 \times 10^5$.

If it is assumed that there are no differences between the molecular weights and the ratios of iodination of the two antibodies, then calculation of the number of sites per cell for the bovine and human cells (A_1B CDe/CDe) gives figures of 5×10^5 and $5{\cdot}5 \times 10^3$ respectively (assuming a molecular weight of $1{\cdot}65 \times 10^5$ and a ratio of iodination (R) of 0·9). As both the molecular weight and the ratios of iodination are at present unknown, it is necessary to repeat the calculation with assumed possible molecular weights and iodination ratios which produce the least difference in the number of sites on the two systems. Thus, changing the molecular weight of the Paul-Bunnell antibody to 1×10^6 and the iodination ratio (R) of the incomplete D Rh antibody to 0·3, the number of Paul-Bunnell sites on the bovine cell is still 5 times that of the D sites on the human cell.

DISCUSSION

The use of ^{131}I as a marker for antibody molecules appears to have many distinct advantages. If the amount of iodine introduced is kept below two iodine atoms per protein molecule the combining capacity of the antibody is apparently not interfered with, and certainly with the two single antibody systems studied no reduction in the serological titre of the antibody could be demonstrated. Even with a greater amount of introduced iodine the reduction in the serological titre was slight.

Although at first sight it might appear preferable to use the ^{131}I in a carrier-free form, this procedure was not adopted for a number of reasons. If carrier-free ^{131}I is used, then the introduction of a quantity of radioactivity of the order of 1 mc into 1 ml. antiserum would mean that only a very small proportion of the total number of antibody molecules would have an opportunity of combining with the iodine. It is the behaviour of these ^{131}I-marked antibody molecules that is studied by measurement of the radioactivity. If there are any slight differences between individual molecules, e.g. in serological behaviour, it is not impossible that these differences might also influence the relative uptake of ^{131}I where the amount of this is strictly limited by competition with other molecules. If in addition there are slight differences in the serological behaviour of ^{131}iodinated and uniodinated antibody molecules the same argument would apply when the combination with cells of these two types of antibody molecule is considered.

If enough carrier iodine is added to give an average uptake of about one atom per protein molecule, then any serological differences introduced thereby are to a large extent common to all the antibody molecules, and the possibility of selective competition is reduced, if not eliminated entirely. It is assumed that the difference between a ^{131}I- and a ^{127}I-marked antibody is altogether too small to detect by the methods employed, and in any case is likely to be far less than the possible difference between a ^{131}I-marked antibody molecule and an unmarked one.

The method of introducing the iodine into the serum proteins which was selected involved the sacrifice of a proportion of the ^{131}I, which for various reasons remained uncombined with the protein. Nevertheless, it was considered more satisfactory to do this than risk creating chemical conditions which might cause alteration to the protein molecules.

The first obstacle which presented itself in the attempt to measure specific ^{131}I-marked antibody on cellular antigens was the non-specific adsorption of ^{131}I-marked serum proteins by the cells. This non-specific adsorption was reduced, as described in series B, by the pretreatment of the cells with unmarked compatible serum before incubation with the ^{131}I-marked sera. It appears that cells always adsorb a certain amount of serum protein on their surface and, by treating them with the unmarked serum first, the amount of non-specific ^{131}I-marked serum adsorbed was reduced to such an extent that it no longer overshadowed the specific ^{131}I-marked antibody adsorption. Nevertheless, the absorption of non-specific material was of considerable interest. Thus, although the amount of human γ-globulin non-specifically adsorbed by Rh-positive and -negative cells was greater than the amount of specific antibody γ-globulin adsorbed by Rh-positive cells, only this latter specifically adsorbed γ-globulin caused the cells to agglutinate in the presence of rabbit anti-human globulin serum.

From the serological aspect, the use of the isotope technique affords a means of establishing the amount of antibody to cellular antigens in a serum and also the number of particular antigen sites per cell. These assessments would be very difficult by any other known method. With cellular antigens, the estimation of small amounts of antibody sensitization by the Kjeldahl technique is not satis-

factory. Kabat & Mayer (1948), in discussing the estimation of antibodies to intact red cells by the Kjeldahl method, state that 'with intact erythrocytes... the amount of antibody N removed is so small a fraction of the erythrocyte N that the method is impracticable'.

It may appear that the amount of antibody protein per ml. serum estimated in both the infectious mononucleosis serum and the Rh antiserum was very small in relation to the respective serological titres. However, it should be remembered that probably single antigen-antibody systems were being measured and not total antibody against all the antigens on a red cell.

The estimation of the number of sites of a particular antigen on a red cell is important because this is obviously a factor which must influence the agglutination of the cells by the corresponding antibody. The finding that the number of Paul-Bunnell sites is considerably greater than the number of D-sites on a human Rh-positive cell (CDe/CDe) offers a reasonable explanation of certain phenomena observed empirically when carrying out serological tests on these two systems. For instance, it is very easy to absorb completely the Paul-Bunnell antibody from a serum and relatively few bovine cells are required. On the other hand, it is much more difficult to absorb the D Rh antibody from a serum and a much larger number of Rh-positive cells is needed. The frailness of the agglutination due to agglutinating Rh antibodies may also be a reflection of the relatively small number of D Rh antigen sites on the cells. With few antigen sites there can be only a small number of antibody molecules binding together the areas of adjacent cells which are in contact in an agglutinate. Finally the relatively few D Rh antigen sites may well account for the very small change in the surface charge of Rh-positive cells when strongly sensitized with Rh antibody (Coombs & Race, 1945).

The authors intend to apply this method to the estimation of the relative number of antigen sites C, D, E, c and e on human red cells. The assessment of the D sites on homozygous (DD) and heterozygous (Dd) cells will also be of great interest.

SUMMARY

1. Experiments have been carried out with antisera marked with ^{131}I in an attempt to study quantitatively the interaction of serum antibodies and red cell antigens.

2. Studies were made on inagglutinable systems. These were 'inagglutinable' bovine cells with ^{131}I-marked rabbit anti-bovine red cell and ^{131}I-marked human infectious mononucleosis sera, and also human cells with ^{131}I-marked human Rh incomplete anti-D sera.

3. The complication due to the non-specific adsorption which interfered with quantitative determinations was avoided by pretreatment of the cells with unmarked serologically compatible serum.

4. The ratio of iodine uptake by the γ-globulin fraction of serum to that of the whole serum was found to pass through a maximum (0·92) with an iodine content of about one atom per protein molecule (assumed average mol.wt. of 10^5).

5. The amount of Paul-Bunnell antibody (in terms of ^{131}I-marked serum protein) in an infectious mononucleosis serum was found to be about 40 μg./ml. serum (7% protein). In the same terms, the amount of D-antibody in an incomplete Rh antiserum was estimated at 54 μg.

6. The number of Paul-Bunnell sites on a single bovine red cell was found to be considerably greater than the number of D sites on a human Rh-positive cell (CDe/CDe). The implication of these results is discussed in the light of some observed serological phenomena.

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The Chemical Composition of the Soluble and Insoluble Fractions of the Bacterial Cell

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Investigation of the chemistry of the bacterial cell wall has been retarded by lack of suitable methods. The results of immunological investigations have suggested that in some organisms its structure is complex, with successive layers of different antigens. Another approach has been through the extraction of whole-cell preparations with organic solvents. Morgan & Partridge (1940) and Freeman, Challinor & Wilson (1940) have extracted polysaccharide-protein complexes from certain Gram-negative bacteria. These substances accounted for about 10% of the whole cell and were believed on immunological evidence to comprise the outer layers. Henry & Stacey (1946) extracted Gram-positive bacteria with bile salt and obtained a magnesium ribonucleate which they considered to be a surface component and responsible for the retention of the Gram stain. Caution must be exercised in interpreting the results of these extraction experiments since it is difficult to establish from which part of the cell the extracted material has come.

Curran & Evans (1942) and King & Alexander (1948) killed bacteria by prolonged shaking with minute glass beads. Dawson (1949) showed that this treatment ruptures the cell wall and allows the contents to escape, leaving the empty shell more or less intact. This seemed to offer the possibility of separating cell walls for chemical study. Salton & Horne (1951*a*, *b*) separated cell-wall material by this method, and also by heat treatment and by treatment with alkali. More recently, Salton (1952*a*) has found that the cell wall of *Streptococcus faecalis* is composed of a protein-polysaccharide complex, similar, perhaps, to those obtained by extraction methods from the Gram-negative bacteria (Morgan & Partridge, 1940; Freeman *et al.* 1940). He suggests that mucoproteins may be important components of bacterial cell walls. Salton (1952*b*) finds that the lipid content of Gram-positive cell walls is low (1–2%) compared with that of Gram-negatives (20%), and there are also differences in the amino acids of the protein component. Mitchell & Moyle (1951) find that three-quarters of the cell-wall material of *Micrococcus pyogenes* is accounted for by a glycerophospho-protein complex which they regard as being probably characteristic of Gram-positive types. Holdsworth (1952) finds that the 'insoluble fraction' of *Corynebacterium diphtheriae* contains 60% protein and 25% carbohydrate. Northcote & Horne (1952) have described certain polysaccharides from yeast cell walls.