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RADIOIODINE LABELED HUMAN IgG AND IgG FRACTION OF RABBIT ANTI-HUMAN IgG SERUM: THE USE OF LABELED ANTIBODY AS ANTIBODY IN PRECIPITATION AND DOUBLE ANTIBODY REACTIONS AND AS ANTIGEN IN PRECIPITATION AND DOUBLE ANTIBODY RADIOIMMUNOASSAYS.

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by

William Alexander Falkler, Jr.

Thesis submitted to the Faculty of the Graduate School of the
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Title of Thesis: Radioiodine Labeled Human IgG and IgG Fraction of Rabbit Anti-Human IgG Serum: The Use of Labeled Antibody as Antibody in Precipitation and Double Antibody Reactions and as Antigen in Precipitation and Double Antibody Radioimmunoassays

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ABSTRACT

Title of Thesis: Radioiodine Labeled Human IgG and IgG Fraction of Rabbit Anti-Human IgG Serum: The Use of Labeled Antibody as Antibody in Precipitation and Double Antibody Reactions and as Antigen in Precipitation and Double Antibody Radioimmunoassays

William Alexander Falkler, Jr., Doctor of Philosophy, 1971

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Radioiodine (^{125}I) labeled antibody has been studied as an antibody in precipitation and double antibody reactions and as an antigen in precipitation and double antibody radioimmunoassays.

Rabbit anti-human IgG serum has been iodinated and the effect of increasing chloramine T reaction time on the percentage radioiodine incorporated studied. The maximum incorporation of radioiodine by the antiserum was 92% and an increase in iodination was proportional to length of reaction with chloramine T in every case but one. Upon electrophoresis the antiserum components displayed the following percentages of radioiodine incorporation: albumin (67-76%), gamma globulins (5-10%), $alpha_{1,2}$-globulins (7-12%), and the beta globulins (5-10%). The differences in radioiodine substitution by each fraction during each increase in reaction time with chloramine T was also recorded.

Isolation and purification of the IgG fraction of rabbit anti-human IgG serum followed by iodination allowed greater incorporation of radioiodine into the antibody molecule. Antibodies at levels of 2.0, 1.7,

1.2, 0.39, and 0.31 atoms ^{125}I/molecule were tested for their activity as antibodies and antigens in precipitation and double antibody reactions under various conditions to promote maximum binding. Only 25% of the labeled antibody would react as an antibody in precipitation tests at these levels of radioiodination, however at these and higher levels (16 atoms ^{125}I/antibody molecule), the antigenic potential of the labeled antibody was not markedly decreased.

Radioimmunoassays were established in which radioiodine labeled human IgG participated as an antigen in a precipitation version with rabbit anti-human IgG serum, and a double antibody version using rabbit anti-human IgG serum and goat anti-rabbit serum. Human serum IgG concentrations were determined using both the precipitation and double antibody radioimmunoassays and the results compared to concentrations determined by gel diffusion techniques. In every case the radioimmunoassay revealed serum concentrations greater than those recorded using the gel diffusion technique.

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INTRODUCTION

"I believe the preparation of any new protein derivative must be considered a research project in itself, one that would require experimentation in preparative procedures and careful characterization of the products, before reliance could be placed in physiological applications." The preceding statement was made by Hughes (1957) in reference to the chemistry of iodination of proteins.

The use of any isotopic tracer technique is based on the premise that the tagged molecule behaves biologically like its unlabeled counterpart. The use of labeled antibodies in serologic reactions has remained a specialized research tool and although some success has been reported, the outcome depended upon allowing a permissible level of iodine incorporation to take place for each antibody studied. The permissible iodine level was found through experimentation to determine the number of iodine atoms per antibody molecule at which the antibody would display its greatest antibody activity.

It is important to study the feasibility of labeling the IgG fraction of rabbit anti-human IgG and IgM serum and reacting it with an IgG or IgM antibody-antigen complex, in this way quantitating the amount of specific antibody in the serum to an etiological agent. A method of quantitating IgG and IgM antibody levels which is less time consuming, less difficult, and more reliable than the current methods employed i.e., 2-mercaptoethanol, ultracentrifugation, and fluorescent antibody techniques would be extremely valuable in evaluating the im-

munological responses to in utero infectious processes. As the review of the literature indicated a lack of such an approach, this research aims to establish a new procedure using radioiodination of the IgG fraction of rabbit anti-human IgG serum and involves the study of the labeled antibody's activity both as antibody and antigen in serologic reactions.

Radioimmunoassays are becoming more prevalent in the field of immunology and serology because of their sensitivity and reliability. The quantitation of serum IgG and IgM antibody is currently performed in most laboratories by a gel diffusion technique. Since in this study the labeled antibody retained its antigenic capacity we attempted also to develop a precipitation and double antibody radioimmunoassay in which the labeled antibody served as antigen in an immune inhibition technique. This technique would allow the quantitation of serum IgG and IgM levels with minimal requirements of isotopic equipment and radioiodine, simple iodination procedures, utilizing commercially available antiserum, and would offer a more accurate and less time consuming means of determining serum antibody levels.

SELECTED LITERATURE REVIEW

Wormall (1930) demonstrated that radioiodine labeled proteins would retain their antigenicity if the level of iodine incorporated into the molecule was not too high. If highly iodinated, proteins lost the majority of their original antigenic specificity and displayed a new specificity due to the presence of iodine in the molecule. Antibody activity was also destroyed with increasing iodination as shown by radioiodination of agglutinins prepared in rabbits (Breinl and Haurowitz, 1932).

A wide variety of plasma proteins have been isotopically labeled and used for various investigative purposes. Iodinated plasma proteins provided a mechanism for studying the "lost plasma" in hemorrhagic, tourniquet, and burn shock (Fine and Seligman, 1944) and anaphylactic shock (Warren and Dixon, 1948). Radioautographic localization of labeled anti-mouse-kidney serum (Pressman, Hill, and Foote, 1949), attempts to saturate the anti-kidney antibody sites in mouse kidney with iodinated anti-mouse-kidney rabbit serum (Pressman and Eisen, 1950), and the in vivo distribution of radioiodinated rabbit immune globulins prepared against rat and mouse tissues (Pressman and Keighley, 1948; Pressman, 1949a & b) were performed by the very active Pressman group. Butement (1949) demonstrated that rabbit antibody to Proteus vulgaris could be absorbed specifically by bacteria when the antibody contained three or fewer iodine atoms/antibody molecule, however its avidity decreased when eight or more atoms were incorporated.

The labeling and study of bovine serum albumin was recorded by Eisen and Keston in 1949. The next year it was shown that substitution of 5-10 atoms of ^{131}I occurred in the tyrosine ring of the serum albumin molecule and that at this level of iodination little or no alteration in the physical properties could be displayed (Hughes and Straessle, 1950).

The rate of iodination of globulins became of interest when Cohen (1950) demonstrated that antipneumococcus antibody iodinates at an equivalent rate as the rest of a globulin fraction, and Pressman and Sternberger (1950) observed that rabbit antibody iodinates at only one third the rate of whole serum and one half the rate of a globulin fraction. Cohen (1951) also demonstrated that impairment of Ab function occurred at iodine levels between 3.5 and 13 atoms/molecule of equine diphtheria antitoxin. However, smaller amounts of iodine produced little or no change in precipitable activity of the antitoxin.

A quantitative precipitin reaction between human serum albumin and its radioiodinated homologous rabbit antibody was developed by Masouredis, Melcher, and Koblick (1951). The amount of radioactivity found in each immune precipitate was proportional to the antibody nitrogen precipitated. Under their iodination conditions (1.3 iodine atoms/antibody molecule) no loss of immunologic specificity was observed.

Iodine was not the only radioisotope to be incorporated into gamma globulins at this time, as much work was carried out with internally labeled phosphorus containing proteins (Libby and Madison, 1947; Banks et al., 1950) and externally labeled sulfated proteins (Friedberg, Walter, and Haurowitz, 1955). Other internal labeling of homologous globulins was recorded using ^{14}C labeled amino acids and observing the

rate of elimination and fate of labeled antibodies when injected into rabbits (Humphrey and McFarlane, 1954a & b).

Labeled anti-human gamma globulin of high isotope specificity was prepared by iodination of antiserum followed by the specific absorption or precipitation of the labeled antibody. Separation of the labeled antibody from the precipitating antigen was performed by elution at an acid pH without carrier exchange or by elution at a neutral or acid pH with carrier exchange (Talmage, Baker, and Akeson, 1954). Several iodination procedures were used in McFarlane's (1956) study in which iodinated plasma proteins were compared to ^{14}C labeled plasma proteins as to their behavior in rabbits. From this study the jet iodination procedure was developed which utilizes N-HCL to liberate iodine and a burette system for rapid mixing of the reactants.

Berson et al. (1956) and Yalow and Berson (1960) introduced the principle of radioimmunoassay into medicine and made it possible to measure small quantities of hormones in blood or urine. The principle of radioimmunoassay as described by Raiti and Davis (1969) is as follows: a hormone will bind with an antibody specific to it and give rise to an antigen-antibody complex called the bound fraction. This bound fraction can then be separated from the unbound or free fraction. The results of bound-to-free ratio are equated to the amount of hormone present, by reading the result from a standard curve previously constructed for that hormone. The radioimmunoassays which were developed for hormones brought interest and techniques to the field of immunoglobulin assays. Radioimmunoassays have been developed for various hormones such as; plasma insulin (Berson et al., 1956; Yalow and Berson,

1960; Goetz et al., 1963; and Genuth, Frohman, and Lebovitz, 1965), human growth hormone (Greenwood, Hunter, and Glover, 1963; Glick et al., 1963; Catt, Niall, and Tregear, 1967b), chorionic gonadotropin and luteinizing hormone (Midgley, 1966), luteinizing hormone (Catt et al., 1968), human follicle stimulating hormone (Cargille, Rodbard and Ross, 1968) and glucagon (Unger et al., 1959 and Grodsky et al., 1961).

Complement was internally labeled and its role in serologic reactions studied (Penn, Haurowitz, and Yenson, 1957). Weiler et al. (1960) developed an inhibition procedure in which the amount of inhibition of precipitation produced by human serum in a rabbit anti-human-globulin labeled -globulin system is compared to the amount of inhibition produced in the same system by a known amount of purified gamma globulin. The antigen labeled was Cutter's purified human gamma globulin.

The effect of various levels of iodination on the localizing activity of rabbit anti-rat-kidney antibodies was reported by Johnson, Day, and Pressman (1960). As iodination increased the localizing activity of anti-kidney antibody and the binding activity of anti-erythrocyte antibody decreased. In every system studied iodination below two iodine atoms/antibody molecule did not affect antibodies requiring only one site for activity i.e., localizing, binding, or hemolytic antibodies, however, with increasing iodination antibody activity decreased rapidly, and with 30 iodine atoms/antibody molecule most of the activity was destroyed. Quantitative determinations of the serum levels of 6.6 S gamma globulins (immunoglobulin G), B_{2A}-globulins and $gamma_1$-macroglobulins were performed by inhibition techniques (Fahey and Lawerence, 1963).

The average serum level of 6.6 S was shown to be 12.63 mg./ml. or 71% of the total serum gamma globulins, B_{2A}-globulins 22% or 3.94 mg./ml. and $gamma_1$-macroglobulins 7% or 1.16 mg./ml. Greenwood, Hunter, and Glover (1963) developed an iodination method which allowed the rapid preparation of 1.5-2.5 micrograms of ^{131}I labeled human growth hormone. Low amounts of carrier-free (^{131}I) iodine (2 millicuries) were allowed to react, without prior treatment, with small quantities of protein (5 micrograms) in a high-yield reaction (approximately 70% transfer of ^{131}I to protein). Chloramine T was utilized, which in water forms a mild oxidizing agent (hypochlorous acid) and converts iodide to iodine which is then incorporated into the tyrosine ring of the protein.

Because of the importance of radioimmunological methods for detection and quantitation of proteins and polypeptides in biological fluids, numerous procedures were developed for separating antibody-bound labeled antigen from the free labeled antigen. These procedures included chromatoelectrophoresis (Yalow and Berson, 1960), ion exchange (Meade and Klitgaard, 1962), double antibody techniques (Morgan and Lazarow, 1963; Hales and Randle, 1963; and Schalch and Parker, 1964), electrophoretic separation (Hunter and Greenwood, 1964), adsorption to charcoal (Herbert et al., 1965), solvent fractionation (Odell, Wilber, and Paul, 1965), gel filtration (Genuth, Frohman, and Lebovitz, 1965 and Haber, Page, and Richards, 1965), and enzyme partition (Mitchell, Collins, and Byron, 1969).

The duration of the oxidation reaction and subsequent release of free iodine became important as this was directly related to the degree of iodination and inversely related to the apparent damage due to oxida-

tion of the iodinated globulin. On this basis Bocci (1964a) studied the different uptake rates of human and rabbit proteins when reacted with different concentrations of chloramine T for various periods of time. No differences in the electrophoretic or autoradiographic patterns of the labeled proteins could be detected when 1.0 mg. of chloramine T was reacted for 60 minutes with 13-31 mg. of tissue-soluble proteins and 0.025-0.5 millicurie of carrier free iodine -131. Bocci (1964b) then compared the biological behavior of guinea-pig serum proteins radioiodinated by the chloramine T method and the same proteins labeled by the iodine monochloride method of McFarlane (1956). The only disadvantage he noted in using the chloramine T procedure (in comparison with the ICl method) was the persistent, slightly higher percentage of ^{131}I tungstophosphoric acid non-precipitable radioactivity in spite of using carrier iodide and a longer ion-exchange column. In 1966 McConahey and Dixon reported a modification of the Greenwood, Hunter, and Glover (1963) iodination procedure which allowed the iodination of microgram or milligram quantities of protein and used chloramine T/protein ratios of less than 1/1000 of that used by Greenwood's group. They labeled 50 immunological proteins and found very little to no detectable denaturation with in vivo half life studies. This method was used by Langenberg and Schlegel (1967) when they iodinated antibody specific to the common strain of the tobacco mosaic virus with ^{125}I. After iodination the labeled antibodies were used for autoradiographic localization of the virus in plant cells.

Since it was observed by Catt, Niall, and Tregear (1967a) that antigens and antibodies could be adsorbed to polymeric surfaces, another

way of removing the free labeled antigen from the antibody-bound labeled antigen was developed. In their study antibody to the specific antigen (to be measured) was coupled to insoluble diazo-polystyrene particles and hence after incubation the removal of the antibody-bound antigen was easily accomplished. They also employed plastic discs coated with antisera as a means of separating the antibody bound antigen from the free antigen (Catt, Niall, and Tregear, 1967b). Following the use of plastic discs, antibody was adsorbed onto the interior surface of plastic tubes and the unbound labeled antigen easily removed by washing the tube, the antibody-bound labeled antigen remaining (Catt and Tregear, 1967). A "sandwich" solid phase radioimmunoassay for the quantitative determination of human immunoglobulins also utilized plastic tubes (Salmon, Mackey, and Fudenberg, 1969). However, in this method antigen was first adsorbed onto the interior surface of isothiocyanate substituted plastic tubes, antibody then bound to antigen, and finally unlabeled or labeled antigen to the antibody. The unlabeled antigen (in this case immunoglobulins) competes for the binding sites of the antibody which decreases the amount of labeled antigen which can bind to the tube. Mann, Granger, and Fahey (1969) devised a technique for detecting small amounts of immunoglobulins which circumvented the problem of separating labeled antigen-antibody complexes by making the antibody insoluble via coupling it to bromacetyl cellulose. Their test proved to be 100 times more sensitive than the ring diffusion test currently used, and they suggested that this technique would be very useful in measuring immunoglobulin components such as the subclasses of immunoglobulin G (IgG).

Labeled immunoglobulins are becoming very widely used in the field of immunological research. Employing labeled antibodies it was demonstrated that the biological ineffectiveness of antineuraminidase antibodies on the hemagglutination activity or on the infectivity of X-7 influenza viruses was not due to lack of combination of the antibody with the virus (Webster, Laver, and Kilbourne, 1968). Radiolabeled purified proteins derived from nurse and lemon shark serum were passively administered to the homologous species for the purpose of investigating the relationship of 19S and 7S immunoglobulin M (Small, Klapper, and Clem, 1970). This study revealed that the serum 7S IgM is neither a precursor nor a degradation product of the 19S IgM and that the 19S IgM is predominantly intravascular whereas the 7S IgM is distributed both extracellularly and intravascularly. The half-life of labeled immunoglobulins IgG and IgM was studied in normal chickens, in chickens rendered hypogammaglobulinemic by early bursectomy and x-irridation and in dysgammaglobulinemic chickens that had bursectomies only (Frommel, Perey, and Good, 1970). The catabolic rate of labeled IgM was demonstrated to be independent of serum levels, whereas the disappearance curve of labeled IgG was related to its serum concentration. Preparation and purification of radioiodine labeled Australia antigen (Au) and the development of a radioimmunoassay was undertaken by Walsh, Yalow, and Berson (1970). They recorded a sensitivity in their assay 100 times greater than can be obtained by the complement fixation procedure. The determination of all classes of serum immunoglobulins in the perinatal period was undertaken using the Single Antibody Millipore Filtration Method (Faulkner and Borella, 1970). This technique uses the method of Borella

(1968) in which the separation of antigen-antibody complexes from free antigen is accomplished by means of a Millipore filter. The complexes are held in the filter and the free antigen is found in the filtrate. Concentrations of IgA as low as 100 nanograms were readily detectable in cord sera and the presence of IgA in all cord sera tested refutes its reported absence by previous investigations of limited sensitivity.

MATERIALS AND METHODS

ANTISERUM, IMMUNOGLOBULINS, AND PLASMA PROTEINS UTILIZED

Rabbit Anti-Human IgG Serum (RAHIgG), H chain specific
Batch #1657K and 1886U
Concentration 1.65 mg./0.1 ml.

Behringwerke Ag.

IgG Fraction of Goat Anti-Human IgG (MFGAHIgG)
Code #61 - 059
Concentration 2.7 mg. Ab/ml.

Human IgG Serum
Lyophilized Code #64 - 145
Liquid Code #64 - 267 Conc. 2.25 mg./ml.
Goat Anti-Rabbit Serum Code #64 - 016 (GAR)
Rabbit Anti-Goat Serum Code $64 - 006 (RAG)
Bovine Serum Albumin Code #81003 (BSA)

Miles - Yeda Ltd.

Pertussis Vaccine (PV) Lot #2GE06

Lilly

Human Pertussis Immune Globulin (HPIG) Conc. 2.5 mg./0.1 ml.

Hyland Laboratories

Human Serum
Brucella abortus Antigen

Maryland State Health Department

Lowry Protein Determination. The Lowry method for protein determination was selected for use because of its sensitivity (10-200 micro-

grams). The protein sample to be measured in a volume of 0.5 ml. was mixed with 5.0 ml. of the following solution: 50 ml. of 2% Na_2CO_3 in 0.1 N NaOH with 1 ml. of 0.5% $CuSO_4 \cdot 5H_2O$ in 1% sodium or potassium tartrate. After 10 minutes at room temperature 0.5 ml. of 33% phenol reagent was added, immediately mixed, and allowed to stand at room temperature for 10 minutes. The resulting solution was poured into a 10 mm. pathlength silica cuvette and the cuvette placed in a Beckman DB spectrophotometer. The optical densities were recorded at a wave length of 600 millimicrons. The recorded readings were then quantitated by comparison with a standard curve which had previously been prepared with known concentrations of purified bovine serum albumin.

Electrophoresis. Electrophoresis of serum components was accomplished using a Gelman 51170 chamber and a Beckman Duostat constant voltage source. The sepraphore III cellulose acetate strips were soaked in Gelman HR buffer until wet and converted to original gel structure. The strips were blotted gently and then the serum sample placed perpendicular to the long axis of the strip by means of a Gelman applicator. The strips were then bridged across and secured to the chamber by means of magnets. The samples were allowed to electrophorese for 45 minutes at a constant voltage (300 volts). At the end of the time allocated for separation, the strips were placed in Ponceau S stain (500 mg. in 100 ml. of 5% trichloroacetic acid) for five minutes. The excess and background stain was removed by washing one minute each in three trays of 5% acetic acid. The strips were then cleared by dehydration in absolute methanol followed by wetting the strips in a solution of 10 - 15% acetic acid in 95% ethyl alcohol for 30 seconds. The strips

were then placed on glass slides and dried at 60° C for 15 minutes.

Fractionation of Antiserum With Ammonium Sulfate. In order to obtain the IgG fraction of rabbit anti-human IgG serum the following procedure in Weir (1967) was utilized at 4° C. One volume of saturated ammonium sulfate (SAS) was added to every two volumes of antiserum. The SAS was prepared by dissolving 550 gm. $(NH_4)_2SO_4$ in a liter of distilled water maintaining a pH of 6.5 with NH_4OH. The SAS was added dropwise to the serum with constant stirring. The mixture was allowed to stand for 10 minutes and then it was centrifuged at 1800 RPM for 10 minutes and the supernatant removed. The precipitate was washed twice with 50 ml. of 50% SAS, centrifuging and discarding the supernatant each time. The sediment was dissolved in 50 ml. of 0.9% NaCl and the globulin fraction again precipitated with 25 ml. of SAS. After washing the precipitate two times with 50 ml. of 40% SAS, centrifuging and discarding the supernate each time it was again dissolved in 50 ml. of 0.9% NaCl. The precipitation with 33% SAS and the washing with 40% SAS was repeated a third time. The gamma globulin suspended in 40% SAS was centrifuged as before, and the sediment dissolved in a desired volume of 0.9% NaCl. The fraction was then placed in a dialysis bag and dialysed against 0.9% NaCl until all ammonium sulfate had been removed as demonstrated when tested with Nessler's reagent.

Preparation of Sephadex Column. The mouthpiece of a 10 ml. pipette was broken off and the delivery end of the pipette packed with glass wool and glass beads. Three grams of sephadex G-50 were placed in 50 ml. of 0.85% saline for 24 hours to allow swelling of the gel. The prepared sephadex was suspended in three changes of a 0.05 M phosphate buffer with

0.15 M NaCl and 0.2% merthiolate. The sephadex and buffer were swirled and poured into the column, the column being rotated to allow uniform packing. A gel bed volume (10-20 ml.) approximately 60% larger than the total volume to be added to the column allowed separation of protein bound iodine and free iodine. The column was then equilibrated with several liters of 0.05 M phosphate buffer. Before each iodination procedure the column was presaturated with 1.5 ml. 5% bovine serum albumin (BSA) followed by elution with 10 ml. of phosphate buffer.

IODINATION PROCEDURES

MODIFICATION OF THE GREENWOOD, HUNTER AND GLOVER METHOD

Into a 15 x 100 mm. glass test tube (reaction vial) was placed 100 microliters (ul.) of antiserum to human G-globulin/IgG (H chain specific), produced in rabbits. Protein determination was done by the Lowry method and 100 ul. of the antiserum was found to contain 7 mg. protein. The reaction vial was kept in crushed ice prior to iodination. To the reaction mixture was added 1-10 ul. of ^{125}I containing 0.25-1.53 millicuries (mCi.). The high specific activity ^{125}I was obtained from Union Carbide Corporation (catalogue #1-125-P-2) in the chemical form of NaI in 0.1 N NaOH (no reducing agent) and in a concentration of greater than 200 mCi./ml. The liberation of iodine was achieved by the addition of 100 micrograms (ug.) chloramine T in a volume of 25 ul. 0.05 M phosphate buffer. The reaction was allowed to proceed 2-5 minutes and then stopped by the addition of 240 ug. sodium metabisulfite in a volume of 100 ul. in 0.05 M phosphate buffer. The reaction mixture was then transferred to the prepared sephadex G-50 column by means of a Pasteur pipette. The

reaction vial was then washed with 100 ul. of a wash solution containing 800 mg. sucrose and 50 mg. KI/5 ml. 0.05 M phosphate buffer. The wash solution was also transferred to the sephadex column after which the transfer pipette and reaction vial were counted for residual activity.

THE McCONAHEY AND DIXON METHOD

The gamma globulin to be iodinated in a concentration of 1-5 mg./4.0 ml. 0.05 M phosphate buffer was placed in a 30 ml. glass beaker containing a small magnetic stirring rod. The beaker was placed in a plastic tray with crushed ice to allow the reactants to remain cold. The plastic tray was then placed on a mag-mix magnetic stirrer and the appropriate quantity of $Na^{125}I$* (0.01-2.0 mCi.) added while stirring. The selected amount of chloramine T (50-100 mg./0.5 ml. distilled water) was then added dropwise. The reaction was stirred for two to five minutes and then terminated by the addition of sodium metabisulfite (50-100 mg./0.5 ml. distilled water). The reaction mixture was then transferred by means of a Pasteur pipette to the prepared sephadex column for separation of free and protein bound ^{125}I.

INVESTIGATORS' MODIFICATION OF THE McCONAHEY AND DIXON METHOD

The gamma globulins to be iodinated in a concentration of 1-15 mg./2.0 ml. 0.05 M phosphate buffer were placed in a 75 x 12 mm. plastic test tube and the tube placed in a beaker of crushed ice to keep the re-

*New England Nuclear 10 mCi./100 ul.
NaI in 0.1 N NaOH (no reducing agent)

actants cold. To the gamma globulin - 0.05 M phosphate buffer mixture was added the appropriate quantity of $Na^{125}I$* (0.01-2.0 mCi.) in a concentration of 10 mCi./100 ul. The test tube was closed by a piece of parafilm. Then after mixing on a vortex mixer, chloramine T (50-100 ug./0.2 ml.) was added by means of injecting through the parafilm with a tuberculin syringe. Another coat of parafilm was added and mixing continued for five minutes. Then sodium metabisulfite (50-100 ug./0.2 ml. distilled water) was added in the same manner as the chloramine T. After the reaction was terminated, the reaction mixture was transferred via a Pasteur pipette to a prepared sephadex column.

Elution and Collection. The eluate from the sephadex column was collected into 75 x 12 mm. plastic tubes containing 1 ml. of 5% bovine serum albumin (BSA). The BSA helped reduce internal radiation damage and also prevented absorption of the eluate to the tube. One to two ml. of eluate were collected into each tube, 20 such aliquots for each iodination. The collected fractions were then counted for one minute in a solid scintillation counter [NaI(Tl)crystal] and the activity of each tube recorded. Two peaks of activity were noted as seen in Fig. 1, the first peak being due to the iodinated protein, the second peak due to the uncombined ^{125}I. The fractions collected at the top of the descending limb of the first peak were utilized as these proved to be the least damaged fractions. By dividing the total activity of ^{125}I utilized (reaction vial + transfer pipette + eluate collected) into the activity displayed in the protein peak, the % of ^{125}I incorporated into the protein could be calculated.

*New England Nuclear 10 mCi./100 ul.
NaI in 0.1 N NaOH (no reducing agent)

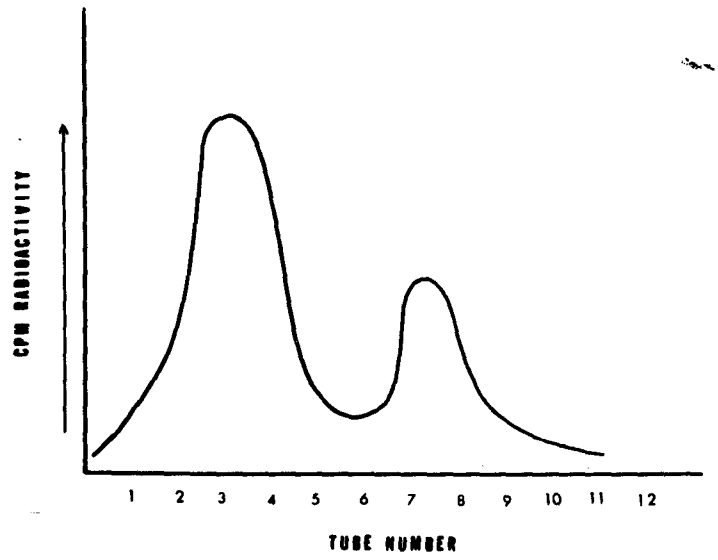

Figure 1

Elution of Radioiodinated Protein from Sephadex Column

Alcohol Precipitation Test. In order to be certain that the activity displayed in the protein peak was due to bound ^{125}I, an alcohol precipitation test was performed in which all the protein and the activity combined with it should precipitate. This was performed as follows: 0.1 ml. of the collected fractions were placed in 75 mm. by 12 mm. plastic tubes with 1.0 ml. of 100% ethanol (done in duplicate). The tubes were mixed and then placed in the refrigerator at 4° C for 18 hours. The activity of the tubes was recorded, then centrifuged at 1700 RPM for 30 minutes, the supernatant removed via a tuberculin syringe and the precipitate and supernatant counted for activity. Free or unbound ^{125}I should not exceed 5%.

Iodination and Electrophoresis of Rabbit Anti-Human IgG Serum. Rabbit anti-human IgG serum (RAHIgG) and pertussis human immune globulin (gamma globulin control) were iodinated by a modification of the Greenwood and Hunter method (1963) using 0.25 mCi. of $Na^{125}I$ (Union Carbide) in every reaction except a five minute reaction in which 1.4 mCi. was utilized. The RAHIgG (Behringwerke - Batch No. 1657K) in a concentration of 7 mg./0.1 ml. 0.5 M phosphate buffer was allowed to react with 100 ug. of chloramine T for various intervals of time (2-5 minutes). The reactions were terminated by the addition of 100 ug. sodium metabisulfite. After elution from a sephadex G-50 column and collection, the activity of the various aliquots was recorded. An alcohol precipitation test was performed and the percentage iodine incorporated calculated. Samples corresponding to each reaction time were placed on cellulose acetate strips and electrophoresed for 45 minutes at 300 volts. The strips were then stained and cut into segments, the segments representing

the various antiserum components. The segments were then placed in plastic tubes and the activity of each fraction recorded using a solid scintillation counter. All procedures were done in duplicate, the percentage radioiodine recorded representing the average.

Binding Tests for Labeled Antibodies. The IgG fraction of rabbit anti-human IgG antiserum (FRAHIgG) was isolated by the ammonium sulfate procedure in Weir (1967). After dialysis and dilution with 0.9% NaCl to the concentration desired (2-15 mg./2.5-4.0 ml.), iodination of the FRAHIgG was performed by our modification of the McConahey and Dixon method with emphasis placed on the number of iodine atoms/antibody molecule incorporated. At iodination levels of 2.0, 1.7, 1.2, and 0.33 ^{125}I atoms/antibody molecule, tests were performed to measure the ability of the labeled FRAHIgG to serve as an antibody in precipitation and double antibody tests. Alcohol precipitation tests were performed on all labeled antibodies and all tests were run in duplicate with saline controls. Control tests were undertaken utilizing unlabeled antigens and antibodies in the approximate concentrations as utilized with the labeled material and all dilutions, unless otherwise stated, were made with 0.9% NaCl. The term binding is used to denote the percentage of activity of radioiodine precipitated.

Binding Test I: Precipitation Test Using Human Pertussis Immune Globulin as Antigen and 2.0 ^{125}I Atoms/Molecule IgG Fraction of Rabbit Anti-Human IgG (FRAHIgG) as Antibody.

Various dilutions (1:10-1:2460) of human pertussis immune globulin (HPIG) in a volume of 0.2 ml. were combined with 0.2 ml. of 2400 counts/minute/0.1 ml. labeled FRAHIgG in 6 x 50 mm. tubes. After addition of

reactants, the tube was sealed by wrapping a piece of parafilm around the mouth of the tube. The contents of the tube were mixed by means of a vortex mixer for five seconds and then the tubes were placed in a water bath-shaker for three hours at 37° C. The tubes were then placed in a refrigerator at 4° C for 18 hours. After incubation, the tubes were counted until at least 4500 counts were recorded. This was followed by centrifugation at 1800 RPM for 30 minutes at 4° C in a clinical centrifuge, the supernatant removed with a tuberculin syringe, and the precipitate again counted for activity. The same incubation and counting procedures were utilized throughout all the following binding tests unless otherwise stated.

Binding Test II: Double Antibody Test Using Pertussis Vaccine as Antigen, Human Pertussis Immune Globulin (HPIG) as Antibody #1 and 2 Atoms ^{125}I/Molecule IgG Fraction of Rabbit Anti-Human IgG (FRAHIgG) as Antibody #2.

Pertussis vaccine (PV) diluted 1:20 in a volume of 0.1 ml. was placed along with 0.1 ml. of various dilutions (1:10-1:2460) of HPIG. The reactants were mixed and incubated as previously mentioned. To the tubes was then added either 6000 counts/minute or 12000 counts/minute labeled FRAHIgG. The tubes were mixed, incubated, the supernatant removed, and counted again by the standard method.

Binding Test III: Double Antibody Test Utilizing Miles Human IgG as Antigen, 2 Atoms ^{125}I/Molecule IgG Fraction of Rabbit Anti-Human IgG (FRAHIgG) as Antibody #1 and Goat Anti-Rabbit Serum as Antibody #2.

Various concentrations of human IgG (100, 50, 25, 12, 6, and 3

ug./0.1 ml. 0.9% NaCl) were placed in 6 x 50 mm. glass test tubes. Approximately 3000 counts/minute/0.1 ml. labeled FRAHIgG was added; mixing and incubation was performed as in the other tests. Then 0.1 ml. of 1:20 goat anti-rabbit serum (GAR) was added and the tubes mixed and incubated as before. Following incubation the tubes were counted, centrifuged, the supernatant removed, and counted as in the other tests.

Binding Test IV: Double Antibody Test Using Pertussis Vaccine as Antigen, Human Pertussis Immune Globulin (HPIG) as Antibody #1, and 1.7 Atoms ^{125}I/Molecule IgG Fraction of Rabbit Anti-Human IgG (FRAHIgG) as Antibody #2.

A. Pertussis vaccine diluted 1:20 in a volume of 0.1 ml. was placed in 6 x 50 mm. glass test tubes along with 0.1 ml. of various dilutions (1:10-1:160) of HPIG. The volume was made to 0.3 ml. with 0.1 ml. of 0.05 M disodium ethylenediamine-tetraacetate (EDTA). Mixing and incubation was performed and then 0.1 ml. of 10,000 counts/minute labeled FRAHIgG was added to the tubes. These tubes were again mixed and incubated, followed by the supernatant removal and counting procedures.

B. In this test the pertussis vaccine was diluted 1:40 and the HPIG diluted (1:10-1:1280). The remainder of the test was performed the same as the A counterpart except for incubation times of two and four days utilized following addition of the labeled FRAHIgG.

Binding Test V: Precipitation Test Using Miles Human IgG as Antigen and 1.7 Atoms ^{125}I/Molecule IgG Fraction of Rabbit Anti-Human IgG (FRAHIgG) as Antibody.

Various dilutions of human IgG (1:10-1:1280) in volumes of 0.1 ml. were placed in 6 x 50 mm. glass test tubes with 0.1 ml. of 0.05 M EDTA

and either 0.1 ml. of 1:100 unlabeled FRAHIgG or saline. Mixing and incubation procedures were performed, followed by the addition of 10,000 counts/minute labeled FRAHIgG to each tube. The tubes were mixed and incubated again, followed by the supernatant removal and counting procedures.

Binding Test VI: Double Antibody Test Using Brucella Abortus as Antigen, Human Convalescent Serum as Antibody #1, and 1.7 Atoms ^{125}I/Molecule IgG Fraction of Rabbit Anti-Human IgG (FRAHIgG) as Antibody #2.

Human convalescent serum serially diluted (1:10-1:1280) in volumes of 0.1 ml. were placed in 6 x 50 mm. glass test tubes with 0.1 ml. of Brucella abortus antigen diluted 1:10. The mixture was incubated in a water bath at 37° C for two days, at which time the tubes were centrifuged at 1800 RPM for 30 minutes, the supernatant removed and the precipitate resuspended in 0.1 ml. 0.9% NaCl. To the resuspended precipitate was added 0.1 ml. of 5000 counts/minute labeled FRAHIgG and incubation proceeded for either 18 hours, or one week. After incubation the supernatant removal and counting procedures were performed as previously described.

Binding Test VII: Precipitation Test With Miles Human IgG as Antigen and 1.21 Atoms ^{125}I/Molecule IgG Fraction of Rabbit Anti-Human IgG (FRAHIgG) as Antibody.

Various concentrations of human IgG (100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, and 0.39 ug./0.1 ml. 0.9% NaCl) were placed in 6 x 50 mm. glass test tubes with various dilutions of labeled FRAHIgG (1, 1:5, 1:10, and 1:20) in a volume of 0.1 ml. The tubes were mixed

and incubated using the standard procedure, followed by the supernatant removal and counting techniques.

Binding Test VIII: Precipitation Test With Miles Human IgG as Antigen and 0.31 Atom ^{125}I/Molecule IgG Fraction of Rabbit Anti-Human IgG (FRAHIgG) as Antibody.

Various concentrations of unlabeled IgG (260, 130, 65, 32.5, 13, 6.5, and 1.3 ug./0.1 ml. 0.9% NaCl) were placed in 6 x 50 mm. glass test tubes with various dilutions of labeled FRAHIgG (1:5, 1:10, 1:20, 1:100, and 1:200) in volumes of 0.1 ml. Mixing and incubation was completed as before and the tubes then counted, centrifuged, the supernatant removed, and again counted as previously mentioned.

Iodination of Commercially Prepared IgG Fractions of Goat Anti-Human IgG (Miles Laboratory) and Its Use as an Antibody. The IgG fraction of goat anti-human IgG serum was obtained from Miles Laboratories (Lot #10) and used without further purification. The Miles IgG fraction of goat anti-human IgG serum (MFGAHIgG) was iodinated by our modification of the McConahey and Dixon method to a level of 0.39 atoms ^{125}I/molecule antibody. Alcohol precipitation tests were performed and all tests were run in duplicate. The following tests were performed to measure the binding ability of the labeled antibody.

Binding Test IX: Precipitation Test Using Miles Human IgG as Antigen and 0.39 Atom ^{125}I/Molecule Miles IgG Fraction of Goat Anti-Human IgG (MFGAHIgG).

Various concentrations of unlabeled human IgG (27, 13.5, 6.75, 2.70, 1.35, and 0.27 ug./0.1 ml., dilutions made with 1:10 normal rabbit serum and saline) were placed in 6 x 50 mm. test tubes with 0.1 ml.

of 1:2 or 1:5 diluted labeled MFGAHIgG. The tubes were mixed and then incubated at 37° C in a water bath-shaker for three hours followed by refrigeration at 4° C for two days. The tubes were then counted, centrifuged, the supernatant removed, and counted again by the standard procedure.

Binding Test X: Double Antibody Test Using Miles Human IgG as Antigen, 0.39 Atoms ^{125}I/Molecule Miles IgG Fraction of Goat Anti-Human IgG (MFGAHIgG) as Antibody #1, and Rabbit Anti-Goat Serum as Antibody #2.

This test was performed exactly as Text IX with the exception that after the incubation procedure, 0.1 ml. of 1:20 rabbit anti-goat serum was added and the tubes reincubated and the procedures for supernatant removal and counting performed.

Binding Test XI: Double Antibody Test Using Pertussis Vaccine as Antigen Human Pertussis Immune Globulin (HPIG) as Antibody #1, and 0.39 Atoms ^{125}I/Molecule Miles IgG Fraction of Goat Anti-Human IgG (MFGAHIgG) as Antibody #2.

Pertussis vaccine 1:10 in a volume of 0.5 ml. was placed with 0.5 ml. of a 1:10 dilution of HPIG and the tubes were placed in a water bath-shaker for three hours at 37° C, followed by refrigeration for three days in order for a heavy precipitate to form. The tubes were then centrifuged at 1800 RPM for 30 minutes, the supernatant removed and the precipitate resuspended in 1.0 ml. of 0.9% NaCl. To the resuspension was added 0.1 ml. of 1:5 diluted labeled MFGAHIgG. The tubes were then mixed and incubated at 37° C in a water bath-shaker for three hours followed by either 18 hours or two days at 4° C. The

activity of the tubes was then counted, the contents of the tube then passed through a 50 millimicron filter as follows.

Method for Filtration of Precipitates: The filter discs were soaked in cold common diluent and before being fitted in place, the filter holder was rinsed with common diluent (CD). The contents of each tube were mixed and delivered to the filter by means of a Pasteur pipette. Suction was applied and the tube washed with two successive portions (approximately 1.0 ml. of ice-cold CD), transferring each washing to the filter. Washing of the filtration apparatus between individual samples was not necessary. One pipette was used for transferring CD to the tubes and another for transferring assay samples and washings from tubes to filter. When filtration was completed, and while the filter was still under vacuum, the glass reservoir was removed from the filter and the filter disc was lifted off of the filter by means of a scalpel blade and forceps. The filtration discs were then placed in 5 cm. square pieces of thin aluminum foil and then transferred to plastic tubes and counted for activity. The empty tubes containing the broken transfer pipettes were then counted and the activity recorded. The activities recorded were corrected for absorption due to the aluminum foil.

Labeled Antibodies as Antigens: The IgG fraction of rabbit anti-human IgM serum (FRAHIgM) was isolated by means of the ammonium sulfate procedure in Weir (1967). Iodination of 4 mg. of the IgG fraction was undertaken by the modification of the McConahey and Dixon method using 0.53 mCi. of $Na^{125}I$. With the incorporation of 45.6% of the radioiodine, the IgG fraction contained approximately 16 atoms ^{125}I/molecule FRAHIgM and was utilized in the following double antibody test.

Binding Test XII: Double Antibody Test Utilizing Miles Human IgG as Antigen, 16 Atoms ^{125}I/Molecule IgG Fraction of Rabbit Anti-Human IgM (FRAHIgM) as Antibody #1, and Goat Anti-Rabbit Serum as Antibody #2.

Approximately 3000 counts/minute of the labeled FRAHIgM in volumes of 0.1 ml. were placed in 6 x 50 mm. glass test tubes with various concentrations of Miles human IgG (100, 50, 25, 12, 6, and 3 mg.). After the standard mixing and incubation procedure, 0.1 ml. of 1:20 goat anti-rabbit serum was added and the tubes again incubated followed by the supernatant removal and counting procedure.

Binding Test XIII: Precipitation Test With 2 Atoms ^{125}I/Molecule IgG Fraction of Rabbit Anti-Human IgG (FRAHIgG) as Antigen and Goat Anti-Rabbit Serum as Antibody.

Labeled FRAHIgG in a concentration of 3000 counts/minute/0.1 ml. was placed in 6 x 50 mm. glass test tubes with various dilutions (1:10-1:11520) of goat anti-rabbit serum. The standard mixing and incubation procedure, followed by the supernatant removal and counting procedure was then performed.

Precipitation and Double Antibody Radioimmunoassay of Human IgG. Purified Miles human IgG gamma globulin (2-4 mg./2 ml. 0.5 M phosphate buffer) was iodinated by a modification of the McConahey and Dixon method using a five minute reaction time with 100 ug. chloramine T and approximately 0.1-0.3 mCi. carrier free $Na^{125}I$ (New England Nuclear). The reaction was terminated by 100 ug. of sodium metabisulfite. After sephadex filtration and elution, alcohol precipitation tests were performed and the % iodination calculated. Approximately 65% of the iodine was incorporated, a level of 2.56 atoms ^{125}I/antibody molecule.

Weiler's (1960) immune inhibition procedure was followed in which a standard system is set up in which the amount of labeled antigen (IgG) and rabbit anti-human IgG serum (RAHIgG) are controlled and the variable factor is the amount of unlabeled IgG added to the system. Precipitation of the labeled antigen will thus be prevented if unlabeled antigen ties up the antibody sites of the RAHIgG.

A standard amount (0.1 ml.) of labeled IgG (3500 cts./min.) was placed along with 0.1 ml. of various dilutions (1:10-1:1280) of Behringwerke RAHIgG (Batch #1866U) into 6 x 50 mm. glass test tubes. The tubes then were mixed by means of a vortex mixer and incubated in a water bath-shaker at 37° C for three hours followed by refrigeration at 4° C for 18 hours. After incubation the tubes were counted until 4500 counts were recorded and then centrifuged at 1800 RPM for 30 minutes at 4° C, the supernatant removed via a tuberculin syringe and the precipitate counted again. The least amount of rabbit anti-human IgG serum (RAHIgG) which gave maximum precipitation of the labeled IgG was chosen for the test.

The second step involved setting up a competition for the seleeted dilution (for example 1:20) of RAHIgG between the 3500 cts./min. labeled IgG and accurately measured amounts of unlabeled IgG. This was performed by adding increasing concentrations of unlabeled IgG (0.054-13.0 ug./0.1 ml.) into 6 x 50 mm. glass test tubes along with the selected dilution of RAHIgG (1:20) and the 3500 cts./min. labeled IgG. Mixing and incubation was performed as mentioned previously. At this point the precipitation and double antibody versions of radioimmunoassay differ. In the double antibody version, after mixing and incubation procedures were performed, 0.1 ml. of 1:20 goat anti-rabbit serum, which precipitated the

rabbit anti-human IgG serum, was added and the tubes again treated to mixing and incubation. In both techniques after the incubation procedures, the tubes were counted, centrifuged, the superntant removed, and counted again in the standard fashion. From the decrease in precipitation of the possible precipitable labeled IgG when varying amounts of unlabeled IgG were placed in the system, standard curves were constructed for both the precipitation and double antibody versions. The effect of using different concentrations of labeled IgG and varied dilutions of RAHIgG on these standard curves was also studied.

Measurements of the IgG concentration of human serum samples were undertaken using gel diffusion (performed by Meloy Laboratories) and both versions of the radioimmunoassays. For use in the radioimmunoassays human serum samples were diluted with 0.9% NaCl to 1:500, 1:750, and 1:1000, and 0.1 ml. of each dilution placed into 6 x 50 mm. glass test tubes. To each tube was then added 0.1 ml. of the selected dilution (1:20) of RAHIgG and 3500 cts./min. labeled IgG. The tubes were then treated to the mixing and incubation procedure after which supernatant removal and counting procedures were performed in the precipitation version. However, in the double antibody version 0.1 ml. of 1:20 goat anti-rabbit serum was added and mixing and incubation procedures again performed followed by supernatant removal and counting in the standard fashion. From the decrease in precipitation of the normally precipitable labeled IgG, the concentration of IgG in the serum sample could be calculated. This is accomplished from the standard curve, the decrease in precipitation of the labeled IgG caused by the diluted serum sample being equated to the decrease in precipitation observed by a known

quantity of unlabeled IgG. This quantity multiplied by the serum dilution will then give the serum concentration of IgG.

RESULTS

Iodination and Electrophoresis of Rabbit Anti-Human IgG Serum.

The percentage radioiodine (^{125}I) incorporated into the rabbit anti-human IgG (RAHIgG) serum when chloramine T was allowed to react from 2-5 minutes is recorded in Table 1. The iodine incorporation increased with increasing chloramine T reaction times in every case except during the three minute reaction. Samples of antiserum from every reaction time were electrophoresed, stained, and the strips cut into four bands (Fig. 2) representing the following antiserum fractions; (1) albumin (2) alpha $_{1,2}$-globulins (3) beta globulins and (4) gamma globulins. The bands were placed in a scintillation counter and the activity of each fraction recorded. The total activity of the four antiserum components was divided into the activity of each individual component, the percentage ^{125}I incorporated into each fraction in this way being calculated (Table 2). Albumin displayed the greatest incorporation of ^{125}I (67-76%) of any fraction during any time interval with chloramine T. The globulins incorporated approximately one fifth the amount of ^{125}I as the albumin fraction. As the chloramine T reaction time increased, the percentage ^{125}I incorporated into the albumin fraction also increased, except during the three minute period (Fig. 3). The percentage ^{125}I incorporated into the albumin fraction with increasing chloramine T reaction times was as follows: 2 minutes, 73%; 3 minutes, 67%; 4 minutes, 74%; 5 minutes, 75%, and 5H minutes (H denotes higher activity 1.4 mCi.), 76%. The alpha$_{1,2}$-globulins displayed the following percentages of ^{125}I incorporation

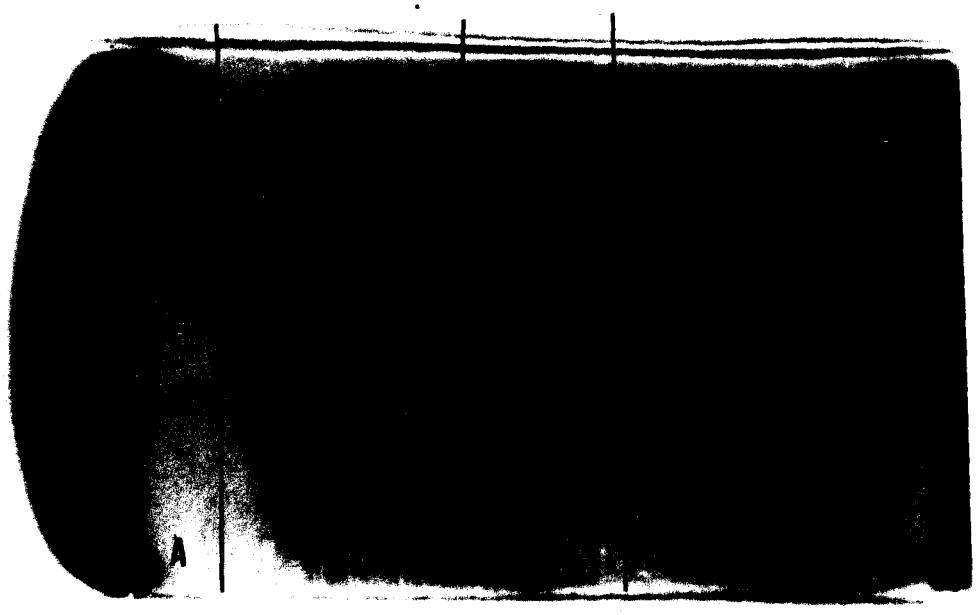

Figure 2

Division of Electrophoresed Strips into Antiserum Components: (A) Albumin (B) Alpha$_{1,2}$-Globulins (C) Beta Globulins and (D) Gamma Globulins.

during the reaction times: 2 minutes, 9%; 3 minutes, 10%; 4 minutes, 7%; 5 minutes, 9%, and 5H minutes, 12%. Incorporation by the beta globulins paralleled that of the alpha$_{1,2}$-globulins and was as follows: 2 minutes, 5%; 3 minutes, 8%; 4 minutes, 6%; 5 minutes, 9% and 5H minutes, 9.4%. In both of these fractions a steady increase in ^{125}I uptake was observed except for the 4 minute reaction time (Figs. 4, 5). The gamma globulin fractions displayed a higher ^{125}I incorporation than the other globulins during the 2 and 3 minute reaction times (Fig. 6), however, after 3 minutes, the ^{125}I uptake decreased progressively as the reaction times increased as follows: 2 minutes, 13%; 3 minutes, 15%; 4 minutes, 13%; 5 minutes, 8%, and 5H minutes, 4.2%. The pertussis human immune globulin displayed 98% ^{125}I incorporated into the gamma globulin fraction and only trace activity in the other fractions.

TABLE 1

CHLORAMINE T# REACTION TIME VERSUS ^{125}I INCORPORATION OF ANTISERUM

REACTION TIME IN MINUTES	% IODINATION
2	80.4
3	70.1
4	85.0
5	89.0
5H*	92.0

#100 micrograms
*H denotes sample iodinated with 1.4 mCi.

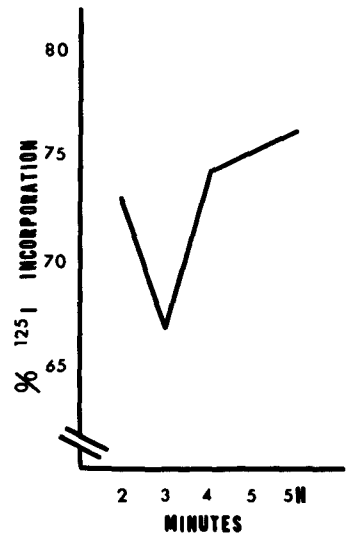

Figure 3

Percentage Radioiodine Incorporation by Albumin Fraction of Antiserum with Increasing Chloramine T Reaction Time (H - denotes higher activity).

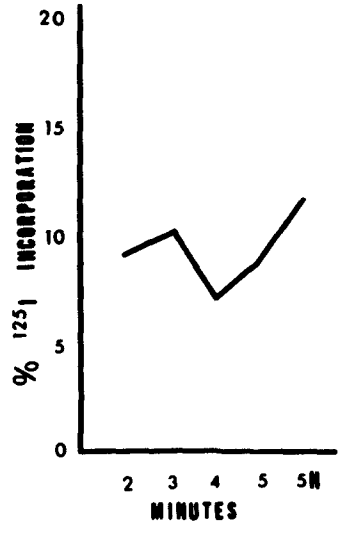

Figure 4

Percentage Radioiodine Incorporation by Alpha1,2-Globulin Fraction of Antiserum with Increasing Chloramine T Reaction Time (H - denotes higher activity).

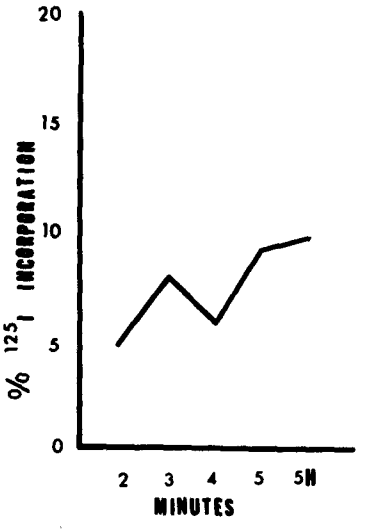

Figure 5

Percentage Radioiodine Incorporation by Beta Globulin Fraction of Antiserum with Increasing Chloramine T Reaction Time (H-denotes higher activity).

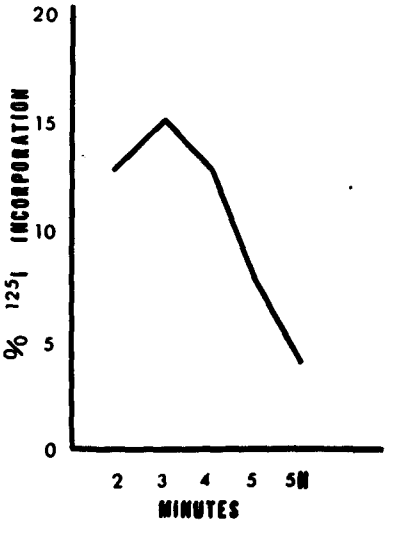

Figure 6

Percentage Radioiodine Incorporation by Gamma Globulin Fraction of Antiserum with Increasing Chloramine T Reaction Time (H - denotes higher activity).

TABLE 2

PERCENTAGE ^{125}I INCORPORATED INTO ANTISERUM FRACTIONS

FRACTIONS	% ^{125}I
Albumin	67-76
$Alpha_{1,2}$-globulins	7-12
Beta globulins	5-10
Gamma globulins	5-15

BINDING TESTS FOR LABELED ANTIBODIES

Binding Test I: Precipitation Test Using Human Pertussis Immune Globulin (HPIG) as Antigen (Ag) and 2.0 Atoms ^{125}I/Molecule IgG Fraction of Rabbit Anti-Human IgG Serum (FRAHIgH) as Antibody (Ab).

In this test the labeled antibody did not precipitate the human pertussis immune globulin (Ag) as no increase in activity was observed in the reaction tubes as compared to the saline control in which no antigen was present. The results of binding test I are seen in Table 3. As precipitation did occur in unlabeled control tests, the question of the labeled antibody binding but not precipitating due to soluble complexes arose. To rule out such a possibility a double antibody test was employed.

TABLE 3

BINDING TEST I: Precipitation Test Using HPIG as Ag and 2.0 Atoms ^{125}I/Molecule FRAHIgG as Ab.

DILUTIONS OF HPIG	AVERAGE % BINDING
1:10	5.0
1:20	5.7
1:40	6.0
1:80	5.8
1:160	4.9
1:320	4.8
1:640	6.9
1:1280	5.8
control (no Ag)	3.4

Binding Test II: Double Antibody Test Using Pertussis Vaccine (PV) as Antigen (Ag), Human Pertussis Immune Globulin (HPIG) as Antibody (Ab) #1, and 2.0 Atoms ^{125}I/Molecule IgG Fraction of Rabbit Anti-Human IgG Serum (FRAHIgG) as Antibody #2.

A visible precipitate was formed in this test, however, this was due to the pertussis vaccine and HPIG forming a precipitable complex. The labeled antibody did not bind to this complex as no activity greater than the saline control was observed with either concentration of labeled antibody (6000 or 12000 cts./min.). The results of binding test II are seen in Table 4.

TABLE 4

BINDING TEST II: Double Antibody Test Using PV as Ag, HPIG as Ab#1, and 2.0 Atoms ^{125}I/molecule FRAHIgG as Ab#2.

DILUTIONS OF Ab#1	AVERAGE % BINDING CONCENTRATIONS OF LABELED FRAHIgG 6000 cts./min.	12000 cts./min.
1:10	7.8	5.0
1:20	7.8	6.5
1:40	7.4	5.1
1:80	6.9	5.8
1:160	6.4	5.3
1:320	6.6	5.7
1:640	6.7	5.5
1:1280	5.1	5.5
1:2560	6.6	6.4
control (no Ab#1)	6.3	6.3

Since the labeled antibody would not bind with HPIG as antigen in the tests performed, binding tests were attempted using a different source of IgG as antigen (Miles human IgG). In order to rule out the possibility of soluble complexes being formed a double antibody technique utilizing goat anti-rabbit serum was utilized.

Binding Test III: Double Antibody Test Utilizing Miles Human IgG as Antigen (Ag), 2.0 Atoms ^{125}I/Molecule IgG Fraction of Rabbit Anti-Human IgG Serum (FRAHIgG) as Antibody #1, and Goat Anti-Rabbit Serum (GAR) as Antibody #2.

The results of this binding test (Table 5) revealed that approximately 27% of the labeled antibody was found in the precipitate at every antigen (IgG) concentration including the saline control in which no Ag was present. Hence the precipitated activity could not be due to the labeled antibody binding with the human IgG and must be due to a precipitation reaction between the goat anti-rabbit serum and the labeled antibody (rabbit origin). In this precipitation reaction the labeled antibody served as an antigen and a constant activity precipitated would be expected as the same amount of labeled antibody and goat anti-rabbit serum was placed in each tube.

TABLE 5

BINDING TEST III: Double Antibody Test Utilizing Miles Human IgG as Ag, 2.0 Atoms ^{125}I/Molecule FRAHIgG as Antibody #1, and GAR as Antibody #2.

Ag CONCENTRATION (ug.)	AVERAGE % BINDING
100	27.2
50	30.1
25	25.6
12	25.4
6	26.1
3	25.9
control (no Ag)	25.7

The apparent lack of antibody activity when 2.0 atoms ^{125}I/molecule were substituted directed our studies to a lower level of iodine incorporation, 1.7 atoms ^{125}I/antibody molecule.

Binding Test IV: Double Antibody Test Using Pertussis Vaccine (PV) as Antigen (Ag), Human Pertussis Immune Globulin (HPIG) as Antibody #1, and 1.7 Atoms ^{125}I/Molecule IgG Fraction of Rabbit Anti-Human IgG Serum (FRAHIgG) as Antibody #2.

The results of part A of binding test IV are seen in Table 6A. The maximum binding occurred at a 1:80 dilution of HPIG and a 1:20 dilution of PV. At these concentrations 17% more activity precipitated than in the saline control.

TABLE 6A

BINDING TEST IV: Double Antibody Test Using PV as Antigen, HPIG as Antibody (Ab) #1, and 1.7 Atoms ^{125}I/Molecule FRAHIgG as Antibody #2

DILUTIONS OF Ab#1	AVERAGE % BINDING
1:10	18.2
1:20	13.4
1:40	21.2
1:80	29.0
1:160	24.9
control (no Ab#1)	11.8

Approximately one fifth of the labeled antibody precipitated, and the same test was performed utilizing greater dilutions of HPIG

and longer incubation times to promote more binding. The results of binding test IV part B are seen in Table 6B. The maximum binding (26.9%) occurred with a 1:80 dilution of the HPIG and a 2 day incubation period. There was no major difference between the 2 and 4 day incubation periods. To be sure the activity precipitated was not due to unbound trapped labeled antibody, the precipitate was resuspended in saline and centrifuged again, no decrease in the activity of the precipitate being recorded.

TABLE 6B

BINDING TEST IV: Double Antibody Test Using PV as Antigen, HPIG as Antibody (Ab) #1, and 1.7 Atoms ^{125}I/Molecule FRAHIgG as Antibody #2.

DILUTIONS OF Ab#1	AVERAGE % BINDING	
	2 DAY INCUBATION	4 DAY INCUBATION
1:10	30.4	25.6
1:20	28.3	32.9
1:40	34.7	31.5
1:80	36.9	32.9
1:160	35.4	32.3
1:320	32.9	35.0
1:640	35.4	33.7
1:1280	30.3	29.4
control (no Ab#1)	10.0	9.0

The binding of over 25% of the labeled antibody using HPIG as antigen initiated the development of a precipitation test using

Miles human IgG as antigen. Unlabeled FRAHIgG was placed in one set of tubes to see if it would compete with the labeled FRAHIgG for binding with the antigen.

Binding Test V: Precipitation Test Using Miles Human IgG as Antigen (Ag) and 1.7 Atoms ^{125}I/Molecule IgG Fraction of Rabbit Anti-Human IgG Serum (FRAHIgG) as Antibody.

The results of binding test V are seen in Table 7. There was no appreciable binding in any of the tubes. As 25% binding was demonstrated with HPIG as antigen and less than 8% in this test, the labeled antibody's binding ability with another type of IgG antigen was studied.

TABLE 7

BINDING TEST V: Precipitation Test Using Miles Human IgG as Ag and 1.7 Atoms ^{125}I/Molecule FRAHIgG as Antibody.

DILUTIONS OF Ag	AVERAGE % BINDING WITH UNLABELED FRAHIgG	AVERAGE % BINDING WITHOUT UNLABELED FRAHIgG
1:10	6.4	6.5
1:20	7.3	8.8
1:40	6.8	7.1
1:80	9.2	7.7
1:160	7.1	12.0
1:320	7.7	8.5
1:640	9.0	11.0
1:1280	10.0	10.0
control (no Ag)	6.4	7.2

Binding Test VI: Double Antibody Test Using Brucella Abortus as Antigen (Ag), Human Convalescent Serum as Antibody (Ab) #1, and 1.7 Atoms ^{125}I/Molecule IgG Fraction of Rabbit Anti-Human IgG Serum (FRAHIgG) as Antibody #2.

The results of binding test VI are seen in Table 8. In this reaction human convalescent serum was reacted with Brucella abortus and a precipitate formed and then the labeled antibody added. Again no appreciable binding greater than the saline control was observed and the labeled antibody apparently lost the ability to bind with Miles IgG and human convalescent IgG, however 25% of the labeled antibody would bind with the HPIG-PV complex.

TABLE 8

BINDING TEST VI: Double Antibody Test Using Brucella Abortus as Ag, Human Convalescent Serum as Ab#1, and 1.7 Atoms ^{125}I/Molecule FRAHIgG as Antibody #2.

DILUTIONS OF Ab#1	AVERAGE % BINDING 18 HOURS INCUBATION	84 HOURS INCUBATION
1:10	5.0	4.7
1:20	5.3	5.2
1:40	6.6	5.5
1:80	4.9	3.9
1:160	4.7	5.5
1:320	3.9	5.2
1:640	5.9	6.9
1:1280	4.9	7.7
control (no Ab#1)	5.0	4.4

The number of atoms ^{125}I/antibody molecule was lowered to 1.2 and an optimal proportion precipitation test was performed in which the antigen (Miles human IgG) and the labeled antibody were both diluted to encompass optimal proportions.

Binding Test VII: Precipitation Test With Miles Human IgG as Antigen (Ag) and 1.2 Atoms ^{125}I/Molecule IgG Fraction of Rabbit Anti-Human IgG Serum (FRAHIgG) as Antibody.

The results of binding test VII are seen in Table 9. A disappointing 6% binding greater than the control was recorded. Having ascertained that the unlabeled FRAHIgG precipitated the Miles human IgG in the approximate concentrations used in the labeled study, another precipitation test was attempted using 0.31 atom ^{125}I/antibody molecule.

TABLE 9

BINDING TEST VII: Precipitation Test With Miles Human IgG as Ag and 1.2 Atoms ^{125}I/Molecule FRAHIgG as Antibody.

CONCENTRATION OF Ag (ug.)	AVERAGE % BINDING DILUTIONS OF LABELED FRAHIgG			
	1	1:5	1:10	1:20
100.00	4.6	4.7	6.1	9.5
50.00	6.0	7.1	8.0	8.3
25.00	5.3	5.0	5.6	7.0
12.50	8.5	4.8	6.1	7.3
6.25	8.4	5.8	6.7	10.2
3.12	8.5	7.7	6.5	6.2
1.56	7.1	7.0	9.0	6.8
0.78	5.4	6.2	6.3	7.2
0.39	3.4	3.5	4.2	2.8
control (no Ag)	2.7	2.6	3.2	2.7

Binding Test VIII: Precipitation Test With Miles Human IgG as Antigen (Ag) and 0.31 Atom ^{125}I/Molecule IgG Fraction of Rabbit Anti-Human IgG (FRAHIgG) as Antibody.

The results of binding test VIII are seen in Table 10. The maximum binding (20.4%) was observed with a 1:5 dilution of labeled FRAHIgG and 13.0 ug. of IgG.

TABLE 10

BINDING TEST VIII: Precipitation Test With Miles Human IgG as Antigen (Ag) and 0.31 Atom ^{125}I/Molecule FRAHIgG as Antibody

	AVERAGE % BINDING DILUTIONS OF LABELED FRAHIgG				
CONCENTRATION OF Ag (ug.)	1:5	1:10	1:20	1:100	1:200
1.3	5.5	7.6	5.2	8.4	10.5
6.3	15.0	17.4	9.0	9.3	10.0
13.0	23.1	9.5	7.6	10.3	12.4
32.5	9.0	8.8	7.6	10.1	11.6
65.0	8.8	8.2	9.6	10.7	12.0
130.0	7.9	7.9	8.9	9.8	14.0
260.0	7.5	8.1	9.3	10.7	12.5
control (no Ag)	2.7	2.4	3.1	2.8	2.7

With only approximately 25% of the labeled antibody participating in precipitation with homologous antigen, commercially fractionated IgG fractions were iodinated and tested for binding to rule out the possibility of some damaging effect of our fractionation procedure which may have inactivated the antibody upon iodination.

Binding Test IX: Precipitation Test Using Miles Human IgG as Antigen (Ag) and 0.39 Atom ^{125}I/Molecule Miles IgG Fraction of Goat Anti-Human IgG Serum (MFGAHIgG) as Antibody.

The results of binding test IX are seen in Table 11. The maximum

binding observed was 12.5% which was recorded using a 1:2 dilution of labeled MFGAHIgG and 2.7 ug. of IgG. The effect of adding a double antibody (rabbit anti-goat serum) was observed in the following test.

TABLE 11

BINDING TEST IX: Precipitation Test Using Miles Human IgG as Ag and 0.39 Atom ^{125}I/Molecule MFGAHIgG as Antibody

CONCENTRATION OF Ag (ug.)	AVERAGE % BINDING DILUTIONS OF LABELED MFGAHIgG 1:2	1:5
0.27	4.7	8.1
1.35	10.5	8.1
2.70	15.3	10.5
6.75	7.9	10.3
13.50	5.5	9.8
27.00	8.4	7.8
control (no Ag)	2.9	3.5

Binding Test X: Double Antibody Test Using Miles Human IgG as Antigen (Ag), 0.39 Atom ^{125}I/Molecule Miles IgG Fraction of Goat Anti-Human IgG Serum (MFGAHIgG) as Antibody (Ab) #1, and Rabbit Anti-Goat Serum (RAG) as Antibody #2.

The results of binding test X are seen in Table 12. The maximum binding (13.4%) was observed with a 1:2 dilution of labeled MFGAHIgG and 1.35 ug. of IgG. The 1:20 rabbit anti-goat serum increased the total percentage activity in the precipitate of each tube, but also raised the activity seen in the control, this being an interaction

of the labeled MFGAHIgG serving as an antigen in a precipitation with the 1:20 rabbit anti-goat serum.

TABLE 12

BINDING TEST X: Double Antibody Test Using Miles Human IgG as Ag, 0.39 Atom ^{125}I/Molecule MFGAHIgG as Ab#1, and RAG as Antibody #2.

CONCENTRATION OF Ag (ug.)	AVERAGE % BINDING OF LABELED MFGAHIgG (Ab#1) DILUTIONS 1:2	1:5
0.27	18.0	21.8
1.35	26.3	25.8
2.70	25.8	20.7
6.75	18.3	20.1
13.50	12.2	15.3
27.00	12.1	11.3
control (no Ag)	12.9	13.2

A test was performed using pertussis vaccine as antigen and human pertussis immune globulin as antibody. The labeled MFGAHIgG would then bind with the Ag-Ab complex and be separated from the free labeled MFGAHIgG by means of filtration through a Millipore filter.

Binding Test XI: Double Antibody Test Using Pertussis Vaccine (PV) as Antigen, Human Pertussis Immune Globulin (HPIG) as Antibody #1, and 0.39 Atom ^{125}I/Molecule Miles IgG Fraction of Goat Anti-Human IgG (MFGAHIgG) as Antibody #2. Separation of Complexes by Millipore Filtration.

The results of binding test XI are seen in Table 13. The maximal

amount of labeled antibody retained on the filter was 10.4% and was observed with 18 hours incubation of a 1:10 dilution of PV and HPIG. This method of separation of bound and free antibody was quite tedious and time consuming and offered no apparent advantage over supernatant removal.

TABLE 13

BINDING TEST XI: Double Antibody Test Using PV as Antigen, HPIG as Antibody #1, and 0.39 Atom ^{125}I/Molecule MFGAHIgG as Antibody #2. Separation of Complexes by Millipore Filtration.

1:10 PV AND HPIG TUBE NUMBER	AVERAGE % BINDING 18 HOURS INCUBATION	48 HOURS INCUBATION
1	11.6	10.6
2	12.3	11.4
3	8.4	9.8
4	6.9	10.2
control (no HPIG)	1.9	3.1

Having observed precipitation tests with the labeled antibody acting as an antigen in several of the double antibody tests, studies using the labeled antibody as antigen were undertaken.

Binding Test XII: Double Antibody Test Utilizing Miles Human IgG as Antigen (Ag), 16 Atoms ^{125}I/Molecule IgG Fraction of Rabbit Anti-Human IgM (FRAHIgM) as Antibody #1, and Goat Anti-Rabbit Serum (GAR) as Antibody #2.

The results of binding test XII are seen in Table 14. The average binding (20.5%) was observed with all IgG concentrations. This demon-

strated that the precipitation reaction occurred even though the labeled antibody contained 16 atoms ^{125}I/molecule. As there was an average of 19% activity precipitated in the saline control the reaction must be with the labeled antibody serving as antigen.

TABLE 14

BINDING TEST XII: Double Antibody Test Using Miles Human IgG as Ag, 16 Atoms ^{125}I/Molecule FRAHIgM as Antibody #1, and GAR as Antibody #2.

CONCENTRATION OF Ag (ug.)	AVERAGE % BINDING
3.0	18.9
6.0	19.8
12.0	20.0
25.0	21.7
50.0	21.0
100.0	20.5
control (no Ag)	19.0

A precipitation test was also performed using various dilutions of goat anti-rabbit serum and a constant amount of labeled antibody as antigen.

Binding Test XIII: Precipitation Test With 2.0 Atoms ^{125}I/Molecule IgG Fraction of Rabbit Anti-Human IgG (FRAHIgG) as Antigen and Goat Anti-Rabbit Serum (GAR) as Antibody.

The results of binding test XIII are seen in Fig. 7. The maximum precipitation was observed with a 1:720 dilution of goat anti-rabbit serum and a normal precipitation curve is observed.

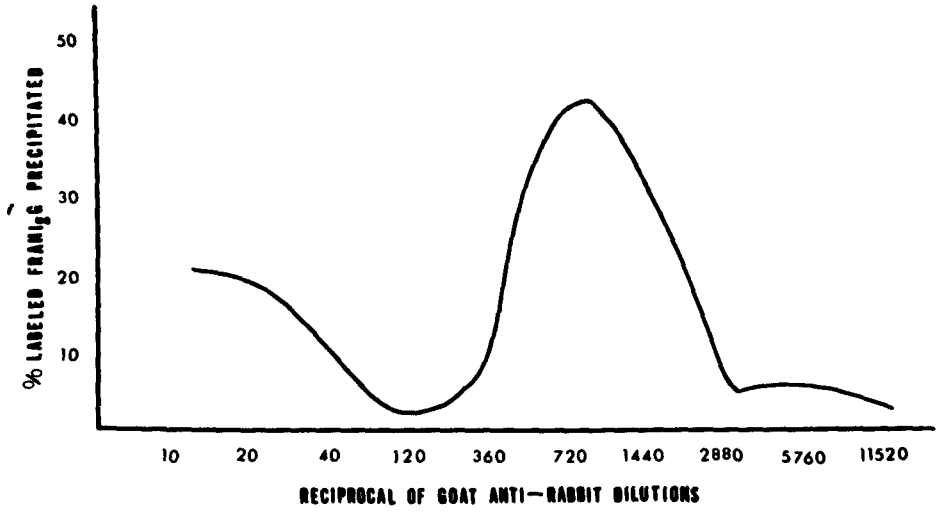

Figure 7

Binding Test XIII: Precipitation Test with 2.0 Atoms ^{125}Iodine/molecule IgG Fraction of Rabbit Anti-Human IgG Serum as Antigen and Goat Anti-Rabbit Serum as Antibody.

Precipitation and Double Antibody Radioimmunoassay of Human IgG.

Fig. 8 demonstrates the reaction between 3500 cts./min. labeled IgG and various dilutions of unlabeled rabbit anti-human IgG (RAHIgG). A 1:20 dilution of RAHIgG precipitated 80% of the labeled IgG and this dilution was used in the following radioimmunoassays.

The standard curves for the precipitation and double antibody radioimmunoassays are seen in Fig. 9. At any particular point, the value of the double antibody radioimmunoassay curve was approximately 0.14 ug. lower than the precipitation curve. It is seen in the precipitation curve that 2 ug. of unlabeled IgG allows (by competition for the antibody sites of the RAHIgG) only 60% of the labeled antigen to precipitate. To use this assay, serum samples must be diluted until they contain between 0.75-3.0 ug. unlabeled IgG in order to compete with the labeled IgG for the antibody sites of the RAHIgG.

Tests were also performed with the precipitation version to determine the effect of different activities (3000, 3500, and 4000 cts./min.) and hence different concentrations of labeled IgG on the standard curves of the radioimmunoassay in which a constant dilution (1:20) of RAHIgG was used. Three different standard curves were obtained and hence three different values for the same serum sample would be recorded if the amount of labeled antigen utilized in the test would be allowed to fluctuate (Fig. 10). The concentration of labeled IgG which displayed 3500 cts./min. was selected for use because it allowed a broader range of usable unlabeled IgG to compete with the labeled IgG for the antibody sites of the RAHIgG.

The effect of a 1:15 dilution instead of the 1:20 RAHIgG dilution

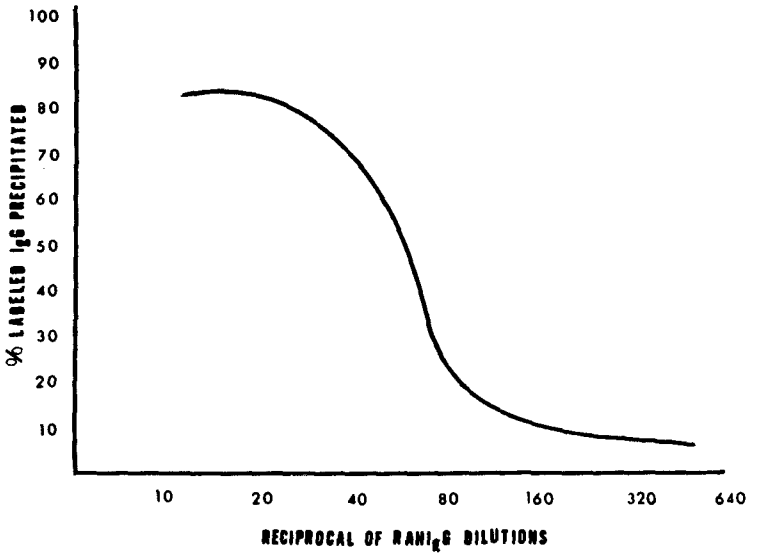

Figure 8

Precipitation Test: Labeled IgG (3500 cts./min.) as Antigen and Rabbit Anti-Human IgG Serum as Antibody.

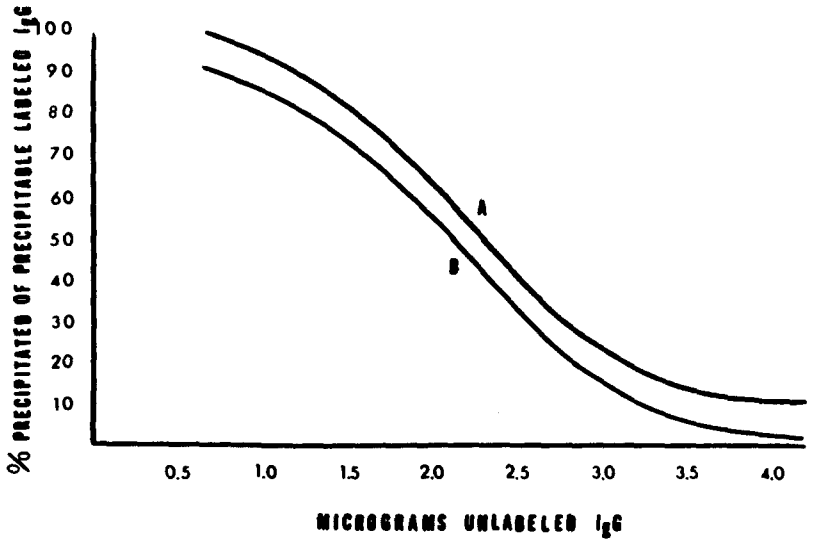

Figure 9

Standard Curves for Precipitation (A) and Double Antibody (B) Radioimmunoassays Using 3500 cts./min. Labeled Human IgG and a 1:20 Dilution of Rabbit Anti-Human IgG Serum.

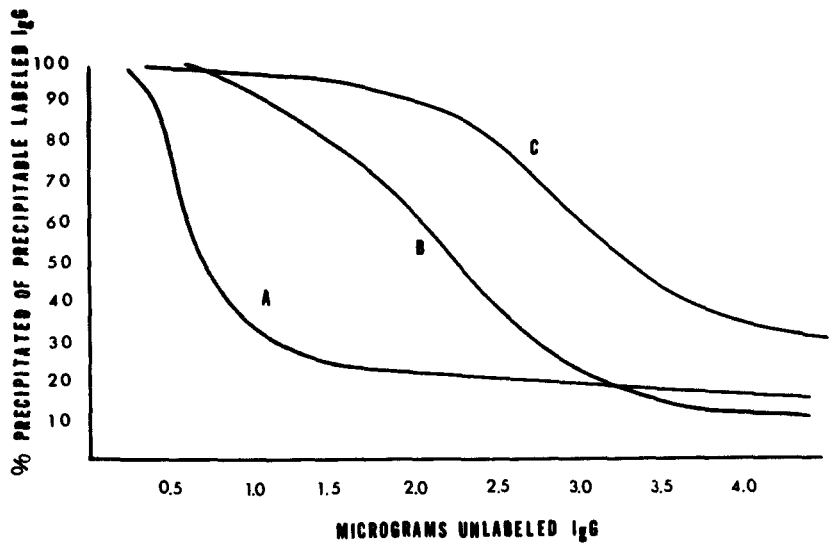

Figure 10

Standard Curves for Three Different Concentrations of Labeled Human IgG (A) 3000 cts./min. (B) 3500 cts./min. and (C) 4000 cts./min. in Precipitation Version of Radioimmunoassay with a 1:20 Dilution of Rabbit Anti-Human IgG Serum.

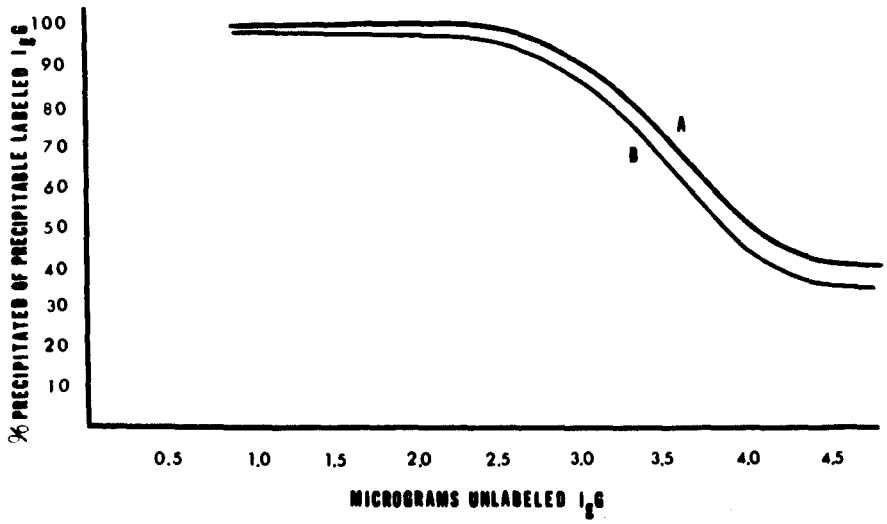

Figure 11

Standard Curves of the Precipitation and Double Antibody Radioimmunoassays Using 3500 cts./min. Labeled Human IgG and a 1:15 Dilution of Rabbit Anti-Human IgG Serum.

on both versions of the radioimmunoassay can be observed in Fig. 11. The slope of the curve is steeper and it can be seen that more unlabeled IgG is needed to compete with the 3500 cts./min. labeled IgG than was recorded with the 1:20 dilution.

Concentrations of IgG were determined on human serum specimens (from Bureau of Labs, State Health Department) by the precipitation and double antibody radioimmunoassays as well as by the quantitative immunodiffusion plate method (Meloy Laboratories). The results of these examinations are seen in Table 15. The serum samples for the radioimmunoassays were diluted 1:500, 1:750, and 1:1000 as these dilutions were found to inhibit approximately 50% of the maximal precipitation of the 3500 cts./min. labeled IgG. The amount of IgG/ml. serum was calculated as follows:

From the decrease in precipitation of the labeled antigen, an IgG concentration can be obtained from the standard curve which represents the amount of unlabeled IgG which causes the same decrease in precipitation of labeled antigen. This value multiplied by the dilution of the serum will give the concentration of IgG in the serum (mg./ml.). For example, let us follow the precipitation assay of serum sample #924. This serum sample was diluted 1:500 with 0.9% NaCl and 0.1 ml. of the dilution was used in the procedure. Only 44% of the labeled antigen precipitated, and in this particular assay 81% of the labeled antigen normally precipitated with no unlabeled IgG in the assay. Therefore 61.6% of the possible precipitable activity precipitated and from Fig. 9 one finds that 2.05 ug. unlabeled IgG also allows only 61.6% of the labeled antigen to precipitate. To find the serum concentration

(mg./ml.), multiply the 2.05 ug. by 500 and then multiply the product by 10 since we originally used only 0.1 ml. of the serum dilution in the reaction (2.05 ug. x 500 x 10 = 10,250 ug. = 10.25 mg./ml.).

From Table 15 one observes that the precipitation reaction in every case gave a slightly higher concentration (0.25-1.30 mg./ml.) of IgG than the amount reported by the diffusion study of Meloy Laboratories. The double antibody version gave serum concentration slightly lower than the precipitation radioimmunoassay in every sample studied, however, these values were still higher than the concentrations reported by Meloy Laboratories.

TABLE 15

QUANTITATIVE DETERMINATIONS OF SERUM IgG#

SAMPLE NUMBER	MELOY LABORATORIES*	PRECIPITATION & DOUBLE ANTIBODY RADIOIMMUNOASSAYS	
917	12.00	12.75	12.57
924	8.95	10.25	10.04
939	14.50	15.25	15.18
950	11.00	11.50	11.25
53307	6.40	7.00	6.80
53308	11.00	11.50	11.25
53407	9.20	12.25	10.10
53443	11.00	11.25	11.20
Avg. Conc.	10.50	11.47	11.05

#(mg./ml.)

*6631 Iron Place, Springfield, Virginia, 22151

DISCUSSION

Iodination and Electrophoresis of Rabbit Anti-Human IgG Serum. Evidently a prerequisite to the use of an ^{131} iodine label for the determination of the absolute amount of antibody in sera would appear to be the determination of the rate of iodination of antibody of the animal species involved (Cohen, 1951).

The technique of iodinating rabbit anti-human IgG serum by the chloramine T method of Hunter, Greenwood, and Glover (1963) followed by fractionation of the iodinated antiserum into components is a feasible method for labeling the IgG fraction of antiserum if large quantities of antiserum and radioiodine are available. The highest incorporation of radioiodine (^{125}I) by the gamma globulin fraction of the antiserum observed in this study was recorded when 7 mg. of the antiserum was reacted with 100 ug. of chloramine T for three minutes. This reaction allows only 15% of the radioiodine utilized to be incorporated into the gamma globulin fraction and only approximately 74% of this activity becomes substituted in the IgG fraction (this calculation made on a weight/100 ml. serum basis and assumes equal iodine incorporation by the immunoglobulin fractions).

There was an increase in the percentage of ^{125}I incorporated into the antiserum with increasing chloramine T reaction time during every interval except the three minute one (Table 1). This is to be expected since increased time allows more production of the active iodine species and hence a greater opportunity for ^{125}I incorporation. Chloramine T is the sodium salt of the N-monochloro-derivative of p-toluene sulphonanide

$\left[(Na^{+})\ [CH_3\langle\bigcirc\rangle SO_2\text{-}N\text{-}Cl]^{-}\right]$, which in aqueous solution slowly yields hypochlorous acid, a mild oxidizing agent. Free iodine is released from $Na^{125}I$ which in the presence of water forms cationic iodine [I^{+}] by the following reaction: $I_2 + H_2O \rightleftharpoons H_2OI^{+} + I^{-}$. Cationic iodine, the active species, is then incorporated into the tyrosine ring of the protein forming monoiodotyrosine and with further saturation, diiodotyrosine. After the tyrosine groups become saturated the active species enter tryptophane and histidine residues. Consideration must also be given to the availability of the incorporating groups to the active iodine species. Tyrosine groups on the surface of the molecule will become iodinated more readily than those within the molecule.

The decrease in the percentage iodination of the antiserum when allowed to react three minutes with chloramine T may be due either to a decrease in the production of the active ^{125}I species or to the inability of the active species to be incorporated into the protein molecule. This reduction in ^{125}I incorporation during the three minute interval with the antiserum also affected the distribution of ^{125}I among the antiserum components, which will be discussed later.

Following electrophoresis of the iodinated antiserum it was readily apparent that albumin incorporated the majority of the active ^{125}I species (Table 2). During all reaction times 67-76% of the radioiodine incorporated by the antiserum was substituted in the albumin fractions, the other electrophoretic fractions substituting the following: $alpha_{1,2}$-globulins, 7-12%; beta globulins, 5-10% and gamma globulins, 5-15%. A comparison of the percentage ^{125}I incorporation by each fraction and the percentage of each fraction in electrophoresed rabbit serum measured by a densitometer

(Carpenter, 1968) was undertaken. Radioiodine incorporations approximately parallel with the reported serum concentrations were observed. The comparative values are seen in Table 16.

TABLE 16

Percentage Incorporation by Antiserum Fractions Versus Serum Concentrations

FRACTIONS	% I INCORPORATED THIS STUDY	% FRACTION OF SERUM CARPENTER (1968)
Albumin	67-76	54.2
$Alpha_{1,2}$-globulins	7-12	8.1
Beta globulins	5-10	12.4
Gamma globulins	5-15	25.3

Although the globulin fractions substituted less ^{125}I than their electrophoretic percentages would indicate, albumin incorporated more than would be expected. This must be due to the availability of the tyrosine rings on the serum albumin molecule. Although rabbit antiserum was used in this study, human serum albumin has been reported to have approximately 18 tyrosines (Hughes and Straessle, 1950) as compared to 60 tyrosines for rabbit IgG (Hughes, 1957). Serum albumin however, is only 43% of the molecular weight of IgG and therefore more molecules and more surface area would be seen per unit weight (4.85×10^{15} more molecules albumin than IgG/mg.). Increased surface area may enhance availability of tyrosine rings to the active ^{125}I species. This

enhanced availability may be the reason for albumin displaying more incorporation than its electrophoretic percentage indicated. Proteins contain a number of readily available reducing groups which are capable of interfering with iodination (McConahey and Dixon, 1966) and albumin may have fewer of these than the globulin fractions.

The globulins demonstrated a lower ^{125}I substitution than would be expected by their serum concentrations. It is extremely difficult to correlate differences in ^{125}I incorporation into the various globulin fraction components as their constituents are varied. The alpha globulins include the transport proteins alpha$_1$ lipoprotein, haptoglobulin, transcortin, ceruloplasmin, alpha$_1$ thyroxine binding globulin, alpha macroglobulin, cholinesterase, lactic acid dehydrogenase, alkaline phosphatase, and the coagulation factors II, IX, V, and X. The beta globulins glycerides, cholesterols, phospholipids, lipid-soluble vitamins, hormones, iron transporting transferrin, some of the complement factors and the haem binding haemopexin (Simmons, Penny, and Goller, 1969). These plasma proteins have varied molecular weights, number of tyrosine groups and available surface tyrosine residues. The gamma globulins are composed of varied chain lengths which result in different molecular weights, group compositions, and structures. Thus it would be impossible to explain the preferential incorporation of ^{125}I into these electrophoretic fractions unless the tyrosine composition, molecular structure, and quantity of each constituent was known.

Bocci (1964b) iodinated rabbit serum with 100 ug. of chloramine T/5 mg. protein allowing the chloramine T to react for 30 minutes. After starch-gel electrophoresis he reported the distribution of radioiodine

incorporated by the serum components. A comparison between his findings and the distribution of radioiodine in the electrophoretic fractions recorded in this study are observed in Table 17.

TABLE 17

Distribution of Radioactivity* in Fractions of Iodinated Antiserum

FRACTION	THIS STUDY (2-5 minutes)	BOCCI'S STUDY (30 minutes)
Albumin	67-76	84.0
$Alpha_{1,2}$-globulins	7-12	4.7
Beta globulins	5-10	3.4
Gamma globulins	5-15	6.6

*Percentage of total activity of serum

Bocci's (1964b) study displayed a greater incorporation of radioiodine into the albumin fraction than we observed in this study which is not surprising as he allowed the chloramine T to react six times longer. The duration of reaction time proportionally increases the active iodine species, however, only so much hypochlorous acid is yielded from 100 ug. of chloramine T. It is probable that the maximum active iodine species was produced before the 30 minute time interval expired as we recorded 76% with a five minute reaction. Again, a preferential substitution into the albumin fraction was noted along with a decrease in incorporation by the globulin fractions as compared to their serum concentrations. In both studies albumin incorporated the most radioiodine followed by the gamma globulins, $alpha_{1,2}$-globulins, and beta globulins.

The three minute reaction, which gave the lowest ^{125}I substitution for albumin, demonstrated the highest substitution level for the gamma globulins (Figs. 3, 6). At three minutes the $alpha_{1,2}$-globulins and beta globulins (Figs. 4, 5) were at a higher level than the four minute reaction. The increased substitution of the gamma globulin fraction during this time interval suggests the three minute reaction time as the best procedure for ^{125}I incorporation into this fraction. A decrease in substitution was observed for the gamma globulin fraction during the five minute and five minute high activity intervals, while all other fractions showed an increase in ^{125}I substitution. The high activity five minute procedure brought about a higher ^{125}I incorporation than the regular five minute reaction, possibly by increasing the speed of the formation of the active ^{125}I species. A greater concentration of $Na^{125}I$ was also available in this case for reaction.

With the chloramine T iodination of rabbit anti-human IgG serum for the purpose of using the labeled IgG fraction of the antiserum in serologic reactions, various intervals of reaction with chloramine T have a pronounced effect on the release of hypochlorous acid and subsequent incorporation of ^{125}I into the tyrosine, tryptophane, and histidine residues of the protein. Not only is the total amount of ^{125}I incorporated into the antiserum varied with the length of reaction, but also differences are observed in the percentage substitution of the various electrophoretic antiserum fractions. If large quantities of radioiodine and antiserum are available, this method is feasible. However, only 15% of the radioiodine utilized will be incorporated into the gamma globulin fraction, this taking place during the three minute re-

action. These results have given us a basic study in the iodination of rabbit anti-human IgG serum and directed our efforts towards isolation and purification of the IgG fraction of the antiserum before iodination, which allows the use of smaller quantities of radioiodine and antiserum and obviates the handling of labeled materials during the fractionation procedure.

Labeled Antibodies Participating in Serologic Reactions as Antibodies and Antigens. The use of labeled antibodies in serologic reactions has remained a specialized investigative research tool because of the differences in antibody activity after iodine incorporation. Before discussing the effects of the incorporation of radioiodine on the binding ability of the antibody, a few permissible radioiodine incorporation levels will be reviewed.

Butement (1949) reported that rabbit antibody when iodinated to a level of 8 atoms of iodine/molecule displayed a decreased ability to be absorbed specifically by bacteria, however, it remained unaffected by three or fewer atoms. The iodination level of 3.5 atoms or less/molecule had no effect on the flocculation of horse antidiphtheria toxin (Cohen, 1951), however, the introduction of 13 atoms of iodine into the antibody molecule decreased the in vitro activity by 20% and the in vivo protective action by about 40%. The precipitating activity of rabbit anti-ovalbumin and of two rabbit anti-hapten sera were decreased with extensive iodination although as many as 18 iodine atoms/Ab molecule did not destroy completely the ability of the labeled antibody to undergo specific precipitation. A level of 1.3 atoms of iodine/antibody molecule was reported as not measurably altering the precipitating activity

of rabbit anti-human serum albumin. The activity of antibodies produced in rabbits against sulfone and ovalbumin did not show any decrease in precipitating activity with eight atoms of iodine/protein molecule (Francis, Mulligan, and Wormall, 1951). A level of 2.7 atoms iodine/protein molecule destroyed the hemolytic activity of rabbit anti-red cell antibody, however, this activity was not measurably affected by 1.8 atoms (Boursnell, Coombs, and Rizk, 1953). An average of 2 atoms of iodine/molecule of antibody did not measurably affect the ability of different types of antibody to precipitate soluble antigens, to bind to erythrocytes, to cause their hemolysis, or to localize in specific tissues, however, increasing iodine incorporation above this level proportionally decreased the antibody activity of these types of antibodies (Johnson, Day, and Pressman, 1960).

Based on the above observations, 2 atoms or less of radioiodine/antibody molecule was chosen as a baseline of iodination in this study. The measurement of antibody activity differs in every study reported so comparison of activity is difficult. In this study only 25% of the labeled antibody participated in complex formation with the homologous antigen which resulted in precipitation. The varied avidity and low number of the labeled antibodies participating in complex formation displayed that more work would need to be undertaken before an assay using a labeled antibody as antibody could be developed.

Why did only 25% of the labeled antibodies participate in precipitable complex formation? Before suggesting answers to this question, we must look at the 6 reactions possible in the chloramine T iodination method as stated by Francis (1960).

(1) Oxidation of certain groupings in the molecule, particularly SH groups:

(2) Substitution of iodine atoms into tyrosine residues to form monoiodotyrosine residues;

(3) Further substitution to form 3-5 diiodotyrosine residues;

(4) Substitution of iodine into histidine residues;

(5) Destruction of tryptophane residues;

(6) Oxidation of cysteine residues to cystine or cysteic acid.

The amount of active iodine species available determines how many of these reactions are completed. The first reaction does not provide the protein with a label, however, it does provide competition for the free active iodine species substitution reactions. Hence, a balance must be achieved in which enough active iodine species are present to counterbalance the first reaction listed, but not enough for reactions 3, 4, 5, and 6 to occur and damage the binding avidity of the antibody.

The activity of antibodies is due to their reactive sites and the hypothesis exists that two are needed for precipitation. The reactive sites of antibodies are located on the outside of the molecule (Kabat, 1968) where they can readily combine with antigens. This external position also offers availability of substitution to the active iodine species. A rabbit antibody molecule contains approximately 60 tyrosines and 16 histidines capable of combining with a total of 136 iodine atoms, however, when 30 atoms were substituted its activity was destroyed (Johnson, Day and Pressman, 1960). Johnson, Day and Pressman (1960) explained this as follows: "This would indicate that groups in the active site or at least the groups required for activity are iodinated more readily than

the average. This might be expected on the basis that tyrosines or histidines in the antibody site would be at the surface on the antibody molecule and more susceptible to iodination than other such sites buried in the molecule." They also mentioned that when small numbers of groups are iodinated the chance of both combining sites on the molecule being substituted is less, and only one combining site is needed for localization, binding, and hemolytic activities.

Another procedure exists for iodinating the antibody at other than the antibody combining sites and this is by tying up of these sites with a hapten prior to iodination. When Pressman and Sternberger (1951) utilized antibodies to simple haptens and iodinated the antibodies when bound to these haptens, the antibody activity remained at levels of iodine incorporation which normally destroyed combining activity. These authors also suggested that the loss of precipitating power of the antibody was due to an alteration of the antibody-specific region during iodination. A destruction of combining power due to a stearic effect upon substitution of tyrosines or histidine residues, or an oxidation of cysteine residues to cystine, or cysteic acid may give a charge effect for repelling the negative group of the antigen. Hence it seems that the antibody specific region is on the surface of the molecule (readily available for the active iodine species), will substitute iodine changing its avidity, and may be affected stearically by charge differentiation if groups near it are iodinated.

The result of iodine incorporation is not the only means by which an antibody can lose its binding ability. One must also consider the oxidative process involved in labeling and its effect on the antibody molecule. The varied means of labeling immunoglobulins demonstrates

the attempts to cause the least damage to the substituted protein (McFarlane, 1956; Greenwood, Hunter, and Glover, 1963; and McConahey and Dixon, 1966). McConahey and Dixon (1966) using a chloramine T method reported minimal damage to 50 immunologically active labeled proteins as shown by in vivo half life studies, however they never utilized their protein derivatives in serological reactions. Apparently the resulting state of our labeled antibody (only 25% of it precipitating) was caused by the iodination procedure as good precipitation was observed in unlabeled control tests after the ammonium sulfate fractionation procedure. Literature review has revealed no other investigation where the IgG fraction of rabbit anti-human IgG serum has been iodinated and utilized in serologic reactions.

Rabbit serum has been iodinated at permissible levels and used in various serologic reactions. It is generally accepted that increasing iodination is directly proportional to loss of antibody activity until at a level of 30-40 atoms iodine/molecule all activity is destroyed. Most of the investigations have reported a level of less than 3 atoms iodine/antibody molecule as not being detrimental to the antibody's activity (Butement, 1949; Cohen, 1951; Masouredis, Melcher, and Koblick, 1951; Boursnell, Coombs, and Rizk, 1953; and Johnson, Day, and Pressman, 1960). The measurement of antibody activity in these studies varies and in our study we are reporting that 25% of all the labeled antibody molecules precipitated in a serologic reaction and not using this 25% as a standard from which the effect of increasing iodination could be studied. It would be very interesting to see our labeled antibody's ability to take part in reactions where only one active site is needed i.e. tissue localizing or hemolytic.

To achieve more participation of the labeled antibodies in complex formation (precipitation), three avenues would be suggested from this research; (1) a less damaging incorporation of iodine, (2) the protection of antibody reactive sites with haptens and hence protecting them from iodination, and (3) internal labeling of the immunoglobulins by feeding an animal labeled amino acids. The major factors considered as variables in the iodination of the IgG fraction of rabbit anti-human IgG serum and its use as a labeled antibody were as follows:

(1) Method of fractionation of antiserum into components.

(2) Concentration of chloramine T.

(3) Length of reaction with chloramine T.

(4) Number of atoms iodine/antibody molecule.

(5) Collection from sephadex column into various diluents.

(6) Diluent containing varied amounts of

a. sucrose

b. carrier KI

c. EDTA

d. phosphate buffer

e. saline

f. protein

(7) Types of antigen and antibodies and concentrations in reactions.

(8) Incubation times and temperatures.

(9) Procedures for separating supernatant from precipitate.

(10) Precipitin and double antibody techniques.

The procedures and results recorded in this study have utilized these variables in selection of the best experimental technique. From the results three suggested avenues have been recorded in order to increase participation of the labeled antibody molecules in binding with human IgG.

Upon performing the serologic tests reported we observed that even the most heavily iodinated antibodies (16 iodine atoms/antibody molecule) would still retain an antigenic capacity. As antigens are polyvalent, more atoms iodine/molecule are apparently permitted to be incorporated without markedly decreasing the ability of the labeled antibody to act as an antigen and form a complex with antibody molecules. This retention of antigenicity after iodination by antibodies allowed the development of a radioimmunoassay for human IgG.

Precipitation and Double Antibody Radioimmunoassays of Human IgG. There have been limited studies demonstrating quantitation of immunoglobulins using radioiosotopes (Weiler et al., 1960; Fahey and Lawrence, 1963; Salmon, Mackey, and Fudenberg, 1969; Mann, Granger, and Fahey, 1969; and Faulkner and Borella, 1970). All of these studies utilized an iodinated antigen and displayed competition between the labeled antigen and unlabeled antigen for a constant amount of antibody reactive sites.

Weiler et al. (1960) is given credit for being the first investigator to perform a procedure which utilized inhibition of labeled immunoglobulin precipitation. He selected a dilution of antiserum which precipitated (90-95%) of the labeled antigen and when plotting his standard curve did not take into consideration that it was only possible to inhibit the activity of the labeled antigen which would precipitate in

the presence of no unlabeled antigen. Although this difference (5-10%) is relatively small, when multiplied by a serum dilution of 500 it becomes significant. Weiler et al., measured only total human gamma globulin as he labeled Cutter's purified human gamma globulin. Fahey and Lawrence (1963) developed a method for quantitating 6.6S gamma globulins, B_{2A}-globulins, and $gamma_1$ globulins in human serum using the immune inhibition technique of Weiler (1960). These investigators labeled their purified globulins by the method of McFarlane (1956) and prepared their own antigens and antisera. They found a serum level of 12.63 mg./ml. as the average normal total serum concentration for 6.6S gamma globulin.

Techniques for the determination of small amounts of immunoglobulins were undertaken utilizing antibody bound to plastic tubes (Salmon, Mackey and Fudenberg, 1969), insoluble antibody (Mann, Granger, and Fahey, 1969), and removal of complexes by filtration (Faulkner and Borella, 1970). Salmon, Mackey, and Fudenberg (1969) used plastic tubes as a means of separating the free antigen from the antibody-bound antigen. Utilizing the principle established by Catt and Tregear (1967) where immunoglobulins would adsorb onto plastic tubes, the investigators first adsorbed antigen onto the tubes, then antibody, and finally labeled or unlabeled antigen forming a "sandwich". Their method was good for quantitation of immunoglobulins in the microgram to nanogram range and hence should be of use in immunoglobulin subclass analysis. The problem in this type of test is controlling the many variables in getting the same amount of antibody to bind to the tubes for each test. We tried a similar technique and were unable to get antibody to bind to plastic tubes

even when the tubes were treated with concentrated sulfuric acid to increase adsorption (Rubin, 1966).

Another way of separating free labeled and antibody-bound labeled antigen was undertaken by Mann, Granger, and Fahey (1969). In order to remove all antibody-bound antigen and not worry about reaching optimal proportions for it to precipitate, the antibody was made insoluble by attaching it to bromacetyl cellulose. The maximum sensitivity achieved by this method was 0.01 ug. IgG, this is indeed more sensitive than gel diffusion and this process takes only one day. The procedure of attaching bromacetyl cellulose to the antibody molecule should be of use in measuring small amounts of immunoglobulins and other proteins in cerebrospinal fluid, ocular fluids, saliva and other secretions as well as subclasses of IgG.

The most recent radioimmunoassay (Faulkner and Borella, 1970) was concerned with the measurements of IgA levels in human cord serum and as reported in the literature review this sensitive method detected IgA in all cord sera tested which refuted the findings that it was absent by previous tests of limited sensitivity. This procedure utilizes the Single Antibody Millipore Filtration Method (Borella, 1968) for separating free labeled antigen from antibody-bound antigen. In this method there are many variables which must be defined for each individual assay i.e., pore size of the filters, protein concentration and nonspecific retention, and incubation time of the antigen-antibody mixture. We tried a modification of this method and found it to be tedious, and offering no apparent advantage over supernatant removal.

The use of immune inhibition techniques of labeled antigen in radioimmunoassays is becoming more prevalent throughout the field of immunology.

We feel the procedures utilized in this study for the quantitation of human IgG could be readily established in the average isotope laboratory and play a role in removing this type of radioimmunoassay from the realm of a specialized research procedure. This precipitation radioimmunoassay takes only one day as compared to two to seven days for the Mancini variety (1965) gel diffusion test, and our procedure utilizes commercially available antiserum and immunoglobulin components. The use of ^{125}I permits the iodinated antibody to be used for several months (60 day half life) and makes practical the use of small amounts of radioactivity. During the decay of ^{125}I, the absence of beta radiation and the smaller amounts of "useless" radiation which the counter will not measure results in a decreased exposure hazard and more efficient counting (Genuth, et al., 1965).

For quantitating IgG in the concentrations occurring in normal human serum our results indicate that a double antibody technique has no apparent increased sensitivity over a precipitation technique. The slightly lower serum concentration of IgG in this version could possibly be due to the goat anti-rabbit serum binding with the rabbit anti-human IgG (RAHIgG) and displacing some of the labeled IgG bound to the RAHIgG, the goat anti-rabbit serum being in a much greater concentration than the labeled Ag.

The procedures utilized in this precipitation radioimmunoassay should also be convenient for the quantitation of IgM and IgA in serum.

SUMMARY

Radioiodine (^{125}I) labeled antibody has been studied as an antibody in precipitation and double antibody reactions and as an antigen in precipitation and double antibody radioimmunoassays.

Rabbit anti-human IgG serum was iodinated by a modification of the Greenwood, Hunter, and Glover (1963) method. Antiserum iodinated with increasing chloramine T oxidation time displayed an increase in percentage ^{125}I incorporation during every reaction but one. The maximal incorporation of the radioiodine observed was 92%. Electrophoresis of the radioiodinated antiserum revealed differences in ^{125}I incorporation by the antiserum components. During all chloramine T reaction times the albumin fraction incorporated the most radioiodine (67-76%), followed in decreasing order by the gamma globulins (5-15%), the $alpha_{1,2}$-globulins (7-12%), and the beta globulins (5-10%). The varied degree of ^{125}I incorporation by each antiserum fraction during each reaction time (2-5 minutes) with 100 ug. chloramine T was also recorded. The low percentage ^{125}I incorporated into the gamma globulin fraction by this procedure suggested that isolation and purification of this fraction before iodination would allow greater ^{125}I incorporation and obviate the handling of radioactive fractions during the fractionation procedure and subsequent dialysis.

After isolation and purification of the IgG fraction of the rabbit anti-human IgG serum, iodinations were performed with emphasis placed on the number of atoms ^{125}I/antibody molecule incorporated.

Antibodies at radioiodine levels of 2.0, 1,7, 1.2, 0.39, and 0.31 atoms ^{125}I/molecule were tested for their activity as antibodies and as antigens in precipitation and double antibody tests. These reactions were employed under various conditions to achieve the maximum participation of the antibody molecules. In all the tests performed utilizing the labeled antibody as an antibody, the maximal binding observed by the labeled antibody never exceeded 25%. Thus, in any of the binding tests employed only 25% of the labeled antibody precipitated with its homologous antigen. However, at these and higher levels of radioiodine incorporation (16 atoms ^{125}I/antibody molecule), the antigenic potential of the labeled antibody was not markedly decreased.

Radioimmunoassays were established in which radioiodine (^{125}I) labeled human IgG participated as an antigen in a precipitation version with rabbit anti-human IgG serum and a double antibody version using rabbit anti-human IgG serum and goat anti-rabbit serum. In these assays labeled and unlabeled human IgG compete for the antibody binding sites of a constant amount of rabbit anti-human IgG serum. Human serum can serve as the source of unlabeled IgG and the IgG concentration of the serum quantitated by its ability to decrease the precipitation of a standard amount of labeled IgG. Values which cause this same decrease in precipitation of the labeled IgG can be obtained from a standard curve previously prepared with accurately measured amounts of unlabeled human IgG. Human serum IgG concentrations were determined using both the precipitation and double antibody radioimmunoassays and the results compared to concentrations determined by gel diffusion techniques. In every case greater serum concentrations were observed employing the

radioimmunoassays and the precipitation version only takes 18 hours to perform. This assay can be accomplished with a minimum of equipment and utilizes commercially available antiserum. This procedure should also be of value in quantitating IgM and IgA.

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