moving electrophoretic components (not shown in Fig. 3). In contrast, these same fractions showed only the 3 or 4 typical slow moving macroglobulin bands (Fig. 3, 3a, 4a) when fractions 3a and 4a were analyzed immediately after gel filtration.

Although passive hemagglutination is a relatively insensitive method for detecting macroglobulin antibody, it lent itself to detection of a significant amount of macroglobulin associated antibody. Those(14) working with natural erythrocyte antigens or bacterial endotoxins adsorbed on erythrocytes have found that immune hemolysis provides enhanced sensitivity in detecting macroglobulin antibody. Under the conditions employed during this study, the macroglobulin associated antibody to soluble protein antigens had no passive hemolytic activity. This particular finding also lent further weight to the specificity of the hemolysin assay for $\gamma_2$-globulin antibody.

Our findings demonstrate the appearance of macroglobulin antibody to soluble protein antigens during primary immune response. The biological activity and physical-chemical characteristics of this macroglobulin antibody remain to be further investigated.

*Summary.* Methods have been described for separating the $\gamma_1$- and $\gamma_2$-globulin antibodies and the macroglobulin antibody of guinea pig sera. Use of simple stepwise elution chromatography on DEAE-cellulose results in extensive purification of $\gamma_2$-globulin antibody. Purified macroglobulin antibody was prepared from relatively large amounts (170 ml) of appropriate sera by combined use of salting out, ion-exchange chromatography and gel filtration.

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## Passive Immune Hemolysis: Titration of Hemolytic Anti-Protein Antibody Using Bis-Diazotized-Benzidine to Couple Antigen to Erythrocytes. (30818)

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Recent advances in the separation of antibody moieties on the basis of their biological activity have decreased the importance of precipitin and hemagglutination determinations of antibodies to the understanding of biological phenomena mediated by immunological mechanisms. The macroglobulin antibodies (IgM) in the case of rabbit and guinea pig precipitate poorly in relationship to their hemagglutination or bacteriophage plaque inhibition titers(1-3), and their biological function remains incompletely defined. The guinea

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pig 7S γ-globulins have been shown to consist of at least 2 distinct species, both of which hemagglutinate antigen-coated erythrocytes and are in part precipitable: the $\gamma_1$ or anaphylactic antibody and the $\gamma_2$ or cytotoxic antibody(4-7).

Benacerraf, Ovary, Bloch and Franklin(5) distinguished between these 2 functionally different 7S globulins in two ways. In starch block electrophoresis, the $\gamma_2$ is slower moving, although complete separation cannot be effected by this means. More important, it became apparent that the $\gamma_1$ antibody is responsible for anaphylaxis and can be detected by the exquisitely sensitive passive cutaneous anaphylaxis titration(8). The discovery that the $\gamma_2$ moiety fixed complement coincided nicely with the demonstration of its cytotoxic activity, as in the case of the reverse passive Arthus reaction(7). Benacerraf and co-workers measured this antibody by adapting the tanned red cell hemagglutination reaction to passive immune hemolysis (7,9,10).

The detection of complement-fixing antibody directed against protein antigens has long been possible by the cumbersome complement fixation test(11). Passive immune hemolysis, where the antigen is placed on carrier erythrocytes, has the obvious advantage of any direct measurement technique. The passive immune hemolysis test using tannic acid treated cells coated with adsorbed protein antigen has the same problems of reproducibility as has its progenitor, the tanned cell hemagglutination reaction. The antigen has been shown to come off the cells(12) and even uncoated tanned cells tend to lyse spontaneously in the presence of complement(13).

Stavitsky and Arquilla reintroduced diazo compounds to hemagglutination techniques based upon the earlier work of Landsteiner and van der Scheer(14) and Pressman, Campbell and Pauling(15). Since their studies with diphtheria toxoid, ovalbumin, and bovine γ-globulin using bis-diazotized-benzidine to couple the antigen in covalent linkage to the indicator red cells(13), several other groups have extended the range of protein antigens and added important modifications (16-18) to this reaction. Stavitsky and Arquilla adapted the same technique to passive immune hemolysis but encountered serious difficulties in spontaneous hemolysis of the treated cells(13,21).

This paper describes a method for detection of the $\gamma_2$ (cytotoxic) antibody of the guinea pig by passive immune hemolysis, based on the foregoing contributions, which is sensitive, specific, reproducible and which overcomes most of the problems encountered by previous workers. The cells prepared in the manner described below may prove to be adaptable to detection of hemolytic antibody produced by immunocompetent cells *in vitro* techniques, such as Jerne plates.

*Materials. Protein antigens and preparation of antigen solutions.* Ovalbumin (Ea), 2 times crystallized (Worthington Biochemical) and bovine γ-globulin (BGG), equivalent to Cohn Fraction II (Armour Pharmaceutical) served as antigens in all experiments. A 0.1% solution in 0.11 M phosphate buffer (see below) was used in the coupling reaction.

*Antisera. Anti-Ea and anti-BGG.* A schedule of hyperimmunization yielded high-titered antisera containing significant amounts of $\gamma_2$ (cytotoxic) antibody as detected by immunoelectrophoresis(19). Adult Hartley strain albino guinea pigs were each injected intradermally and in the footpads on day 0 with 2.5 mg of the antigen in 0.5 ml of complete Freund's adjuvant (containing 2.5 mg heat-killed *M. tuberculosis,* Hv 37). On the 21st day 0.5 ml of incomplete adjuvant containing 2.5 mg of the antigen was distributed intradermally in the nuchal region only. On the 27th day 0.1 ml of a 1% antigen solution (in saline) was injected intradermally in a single flank site. The animals were exsanguinated under ether anesthesia on day 35, the sera separated after overnight clotting at 5°C, then pooled and stored at −10°C. Before assaying for hemagglutination or hemolysis titers, aliquots to be analyzed were decomplemented at 56°C for 20 minutes and then adsorbed with equal volumes of packed washed normal sheep erythrocytes for 10 minutes at room temperature.

*Fractionation of antisera for PCA ($\gamma_1$) and hemolytic ($\gamma_2$) 7S antibody.* Diethyl-

aminoethylcellulose (DEAE-cellulose) ion-exchange column chromatography, as described elsewhere(20), provided separation of guinea pig $\gamma_2$ from $\gamma_1$ antibody through a simple 2-step gradient. 0.01 M potassium phosphate buffer (pH 8.0, 0.01 M) brought off the $\gamma_2$; the $\gamma_1$ came off with the remaining serum proteins in the 0.3 M potassium phosphate buffer (pH 8.0) eluate. Appropriate fractions were pooled and concentrated by ultrafiltration in the cold (4°C) to the *volume* of the aliquot of the original whole serum chromatographed and then titrated for hemagglutinating, hemolytic and PCA activity.

*Erythrocytes for coupling reaction.* Healthy normal sheep maintained at the NIH animal farm were bled weekly and the whole blood was stored in Alsever's solution at 5°C. Each test day an aliquot sufficient for adsorption and reaction needs was removed and washed 3 times in phosphate-buffered normal saline, pH 7.4 (ratio of 10 volumes of buffer to 3 of Alsever's) in 40 ml conical centrifuge tubes (at 7°C under 500 × *g* for 10 minutes). No cells older than one week were used. We have no experience with older cells (*cf.*, Mayer's chapter on Complement in (11)) or cells of other species. (Other workers with BDB in hemagglutination systems have specified fresh erythrocytes(17,21).)

*Preparation of stock bis-diazotized-benzidine solution (BDB).* 0.643 g of benzidine dihydrochloride was added to 88.0 ml distilled water and 2.2 ml of a 6 Normal hydrochloric acid solution and allowed to dissolve at 4°C overnight. With room temperature at 4°C and the reactants on ice, 0.350 g of sodium nitrite (previously dissolved in 10.0 ml distilled water) was added and the mixture stirred intermittently for 30 minutes with an occasional test for free nitrous acid with starch iodide paper. After this period, tests at one-minute intervals were made until the lengthening of the time of onset of test paper coloration stabilized at a very slow rate. The reaction was then considered to be complete. One milliliter volumes of the clear straw-colored solution were then immediately quick-frozen (dry ice-acetone bath) in screwcap vials and stored at —60°C. Subsequent handling always involved use of ice baths and ice-cold buffers to prevent degredation (indicated by brownish discoloration and cloudiness) (11,13,16).

*Preparation of buffers.* A calcium-magnesium stock solution contained 3.320 g anhydrous reagent grade $CaCl_2$ and 20.332 g $MgCl_2 \cdot 6H_2O$ made up to one liter volumetrically with distilled water. A stock 5-fold concentrated Tris complement buffer (TCB) contained 25.00 ml of the Ca-Mg stock solution, 200 ml of 1 Normal hydrochloric acid, 30.285 g of Trishydroxymethylaminomethane (Tris), and 28.580 g of NaCl, made up to one liter volumetrically with distilled water. This buffer was diluted 1:5 with distilled water and the pH adjusted to 7.32 with 1 N HCl. The 0.11 M photphate buffer, pH 7.4, was made with 4.433 g $NaH_2PO_4 \cdot H_2O$ (monobasic) and 19.458 g $Na_2HPO_4$ (dibasic) made up to one liter volumetrically with distilled water. This buffer was used in all aspects of dilution of antigens, BDB stock, and erythrocytes. For dilution of antisera in hemagglutination reactions, it contained 1% normal rabbit serum. The diluted TCB replaced the phosphate buffer in the case of dilution of antisera for hemolysin titration and in the final suspension of the antigen-coupled erythrocytes for this assay. Buffers were kept ice-cold at all times.

*Experimental and results.* Hemagglutination was used to establish the proper protein-to-BDB ratio for satisfactory coupling of the antigen to the indicator erythrocytes. The proportions thus obtained were then extrapolated to preparation of large batches of antigen-conjugated cells which were then examined for their susceptibility to autohemolysis in various buffers. The Tris complement buffer proved to minimize autohemolysis yet was not anti-complementary. Subsequently quantitative techniques were applied to the hemolytic technique. Specificity for antigen and species of antibody was then evaluated and reproducibility and sensitivity studies conducted. These experiments are reported in detail below.

*Determination of the proper protein : BDB ratio by hemagglutination.* Following in general methods previously described(13,17), we

TABLE I. Determination of the Best BGG:BDB Ratio for Hemolysin Cells.

| Row | BGG/BDB ratio (mg protein per 0.25 ml of 1:15 BDB solution) | ml of 0.1% BGG solution | ml 0.11 M phosphate buffer | Hemagglutination titer of standard anti-BGG (reciprocal) | Quality of negatives (agglutination pattern) | Degree of autohemolysis (at room temp) after 24 hrs (visible coloration supernate) |
|---|---|---|---|---|---|---|
| A | .25 | .25 | 2.75 | 128,000 | Fair* | Slight |
| B | .50 | .50 | 2.50 | 64,000 | " | " |
| C | .75 | .75 | 2.25 | 64,000 | " | " |
| D | 1.00 | 1.00 | 2.00 | 32,000 | " | " |
| E† | 1.50 | 1.50 | 1.50 | 32,000 | Excellent | None |
| F | 2.00 | 2.00 | 1.00 | 16,000 | " | " |
| G | Control: normal erythrocytes | | 3.00 | 0 | " | " |

* In the highest negative dilution, the button had a small central pinhole which expanded in the lower negative dilutions.

† These cells combined button-like negatives, sharp transition zone (not shown), and resistance to autohemolysis with an acceptable hemagglutination titer (*cf.* to tanned cell hemagglutination) for a hyperimmune antiserum.

determined by use of the hemagglutination system the protein:BDB ratio which yielded maximum cell stability, perfect negatives with control sera and high titers with specific hyperimmune antisera. The macro-methodology(10,22) proved to be more sensitive for this purpose than microtiter techniques(23).

The total reaction volume for preparation of coupled erythrocytes for hemagglutination was fixed at 3.35 ml of which 0.1 ml was from a 50% suspension of 3-times washed normal sheep erythrocytes in 0.11 M phosphate buffer and 0.25 ml from the diluted BDB solution (1:15 in cold phosphate buffer). The only variables were the amounts of 0.1% antigen solution and the additional phosphate buffer required to bring the mixture to volume. An example is shown in Table I. (It was sometimes necessary subsequently to vary the amount of BDB solution while holding total amount of antigen constant)(13,16,17.)

The sequence of addition of reagents and subsequent washes was as follows. The erythrocytes and appropriate quantities of cold antigen solution and buffer were diluted into a 15 × 150 mm round bottom test tube. The tube was then placed in an ice bath and both were rotated for 10 minutes while the BDB stock solution thawed and then diluted. The freshly diluted ice-cold BDB then was added to the other reactants and the mixture gently agitated on the rotator at room temperature for exactly 30 minutes. The product was centrifuged for 10 minutes at 200 × *g* in the cold (7°C), the yellow supernatant discarded, and the cells carefully and completely resuspended in 3-5 ml of cold phosphate buffer. Two additional washes were carried out. After the last wash, the packed cells were resuspended in 2.5 ml of a 1% normal rabbit serum (decomplemented and adsorbed) phosphate buffer solution. The cells thus prepared were used during the same day. A similar suspension of normal sheep erythrocytes constituted control cells.

Titration of a heated and adsorbed known high-titered antiserum was performed using appropriate two-fold dilutions in the 1% normal rabbit serum phosphate buffer. Each tube contained 0.50 ml of serum dilution and received 0.05 ml of the antigen-coated erythrocyte suspension(22). The agglutination was controlled for specificity by one tube containing 1% normal serum plus antigen-coupled cells and another tube with the lowest dilution of the antiserum together with normal sheep erythrocytes. After careful mixing of cells and serum dilutions, the reactants stood at room temperature for 48 hours. Agglutination patterns were read after 18 hours and the cells observed for autohemolysis thereafter.

The best protein:BDB ratio was defined by applying the following criteria: (1) its cells demonstrated button-like negatives in control sera and in dilutions of the antiserum beyond the positive zone; (2) a sharp transition (1-2 tubes) from positive to negative occurred in the transition zone; (3) there was

no detectable autohemolysis after 24-48 hours at room temperature; and, (4) of the several ratios exhibiting the first 3 characteristics, this ratio gave the highest hemagglutination titer with the standard antiserum and was comparable to results obtained with tanned cell hemagglutination. Table I illustrates the choice of a ratio based on these criteria for the case of bovine γ-globulin (BGG). This protein:BDB ratio of 1.50 (mg protein per 0.25 ml diluted BDB) was also optimal for ovalbumin (Ea). The Table also illustrates the tendency to spontaneous hemagglutination encountered with low protein:BDB ratios.

*Resistance to autohemolysis in various buffers.* After choosing a particular protein: BDB ratio as most suitable by the above criteria, the resistance of these cells to autohemolysis was determined for 3 different buffer systems: the usual modified barbital buffer for hemolysin titrations(11,22), Tris complement buffer, and 0.11 M phosphate buffer, pH 7.4. Several times the usual quantity of cells necessary for hemagglutination was prepared, divided into 3 equal parts and placed in 100 ml of each buffer at room temperature. Within a few hours, ovalbumin-BDB-cells in modified barbital buffer underwent some lysis while over this period the same cells in Tris complement buffer and phosphate buffer remained stable. After 24 hours at room temperature, the antigen-coupled cells in modified barbital buffer had hemolyzed completely, those in Tris complement buffer had partially hemolyzed, while the cells in phosphate buffer remained intact. Because of the anti-complementary nature of phosphate ion (11), the Tris buffer was chosen for use with the conjugated cells in subsequent hemolysin titrations. It was not suitable for hemagglutination reactions since they were read after standing overnight. This buffer proved to give the same data as modified barbital buffer for a standard rabbit anti-Forssman hemolytic antiserum.

*Preparation of cells for hemolysin titrations.* The technique of hemolysin titration employed was the 50% hemolysis method described by Taliaferro and Taliaferro(24, 25) as modified by Campbell *et al*(22). Extrapolation of the protein:BDB ratio from hemagglutination requirements to the hemolysin test took into account the need for strict quantitative techniques in preparation of the 1% erythrocyte suspension utilized in this reaction. Reproducibility proved to depend on addition of accurate amounts of antigen and diluted BDB solutions and erythrocytes to the reaction mixture and on subsequent exact adjustment of the antigen-coupled cell suspension to the proper concentration.

The detailed method which follows assumes a protein:BDB ratio (mg protein per 0.25 ml 1:15 dilution of BDB solution) of 1.50 as optimal, which was the case in these experiments with ovalbumin and bovine gamma globulin. *In coupling the antigen to the cells, the relative volumes of antigen and BDB solutions and phosphate buffer and the concentration of erythrocytes in the latter must be adjusted appropriately in the case of other antigens having different optimal ratios.*

The following method was used to prepare approximately 80 ml of a 1% suspension of antigen-coupled cells; the amounts of reactants can be doubled without altering the quality of the final product. A 3.3% suspension of washed normal sheep erythrocytes was prepared by adding 7.0 ml of a 50% suspension to 100.0 ml of 0.11 M phosphate buffer. Final adjustment of concentration was made to an optical density of 0.500 ± 0.004 (at 530 mμ on a Beckman D.U. Spectrophotometer using cells with a 1 cm light path): three 1.0 ml aliquots of the 3.3% suspension were each diluted with 9.0 ml of distilled water, the O.D.'s determined and averaged, the volume adjusted accordingly and checked.

An aliquot of 30.0 ml of the 3.3% erythrocyte suspension was delivered into a 250 ml Erlenmeyer flask and gently mixed in an ice bath on a rotator for 20 minutes with 30.0 ml of the 0.1% protein solution (Ea or BGG). Meanwhile the 1:15 BDB solution in cold phosphate buffer was prepared and then 5.0 ml added to the reaction flask. The flask was removed from the ice bath and rotated at room temperature for exactly 30 minutes. The suspension was then poured into four

40 ml 28 × 115 mm round bottom centrifuge tubes and centrifuged (200 × *g*) in the cold (7°C) for 12 minutes. The supernatant, which is yellow in the first wash, was decanted and the packed cells completely resuspended in a total of 60 ml of cold phosphate buffer. After 2 additional washes, the cells were resuspended in 60 ml of Tris complement buffer and this suspension was adjusted to 1% as follows. Three 1.0 ml aliquots of the TCB suspension were each lysed with 4.0 ml of distilled water and the O.D. at 530 mμ determined. Volume adjustments were then made to bring the final concentration (measured in this way) to an O.D. of 0.300 ± 0.002 and then checked.

Completion of the preparation of the antigen-coupled cells was timed to coincide with completion of dilutions of the various sera to be tested. The cells were kept ice-cold until delivery into the hemolysin tubes. The various reagents were added to the serum dilutions in the following manner: 13 × 100 mm test tubes containing in 1.0 ml appropriate quantitative dilutions (or buffer blank) of heated and adsorbed antiserum TCB received 2.0 ml of the cold 1% erythrocyte suspension. The reactants were well-mixed by inversion and allowed to stand for 10 minutes at room temperature. Then 2.0 ml of a cold complement dilution in TCB, containing 2 50%-units/ml, was added.† The reactants were mixed again by inversion and then immediately placed in a 37° water bath for a 30-minute incubation. The tubes were then removed and immediately centrifuged (1200 × *g* for 20 minutes) in the cold (7°C) to stop the hemolytic reaction. The supernatants of the 3 blanks were read at 530 mμ against distilled water as an index of the degree of autohemolysis (which should not exceed 5% = O.D. 0.030). The slit width was then adjusted to give the blanks an O.D. of 0.000 and the O.D. of the supernatants of the various antiserum dilutions determined. The titers were calculated by application of the van Krogh equation; on arithmetic graph paper, the value $\log\left(\frac{\text{O.D.}}{.600-\text{O.D.}}\right)$ was plotted against the log of the dilution and the intercept at O.D. ascertained. Rejection of O.D. values below 0.090 and above 0.520 followed the method in Campbell *et al*(22), which also details the various types and sizes of pipettes necessary to enhance accuracy.

*Results of hemolysin titrations: specificity, sensitivity, reproducibility.* Several standard hyperimmune guinea pig anti-Ea and anti-BGG antisera and a variety of normal sera from 3 different strains of normal guinea pigs (Sewall Wright Strain 2, Strain 13, and Hartley strain) were tested for hemolytic activity against ovalbumin-conjugated erythrocytes and bovine gamma globulin-conjugated erythrocytes. The upper graph in Fig. 1 shows that normal sera fail to hemolyze sensitized cells while the specific antiserum has a titer

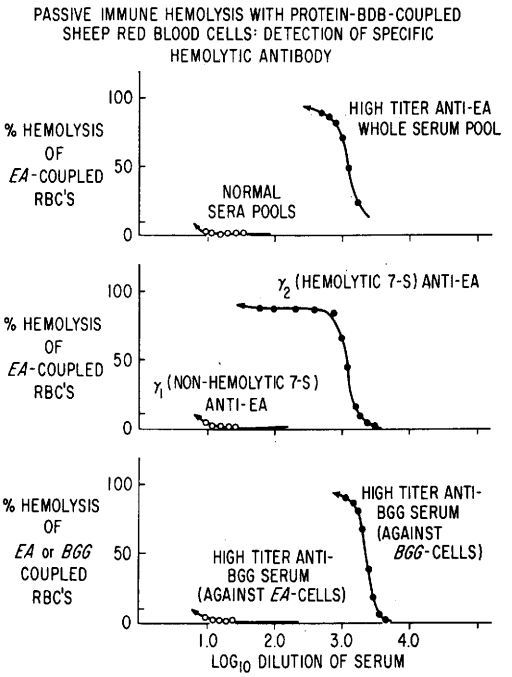


FIG. 1. Graph A compares the titer of several individual normal serum pools (grouped as one) and a specific hyperimmune antiovalbumin antiserum containing $\gamma_2$ antibody in their ability to lyse antigen-coupled cells. Graph B represents the serum shown in A after fractionation into $\gamma_1$ and $\gamma_2$ moieties and compares their ability to lyse the antigen-coupled cells. Graph C shows that hyperimmune hemolytic anti-BGG antiserum can hemolyse only BGG-conjugated cells and not Ea-conjugated cells.

† Complement was titrated by the same method using a system of 2 50% μ/ml of a standard rabbit anti-Forssman antiserum and normal sheep erythrocytes(22).

TABLE II. Sensitivity of the Hemolysin Assay for Detection of $\gamma_2$ Antibody.

| Source of 7S species | Hemagglutination titer (reciprocal) | Hemolysin titer (50% units/ml) | Hemolysin sensitivity (*cf.* hemagglutination) |
|---|---|---|---|
| Column IV-$\gamma_2$ anti-Ea | 20,000 | 1124 | = 1/20th |
| Column XII-$\gamma_2$ anti-BGG | 16,000 | 2215 | = 1/10th |

of 1124 50% units per ml. The bottom graph compares the hemolytic activity against BGG-conjugated cells of an anti-BGG serum with the same anti-Ea serum seen in the upper graph. The specific antiserum has a titer of 3000 50% units per ml while the anti-Ea serum fails to hemolyze the BGG cells.

Having demonstrated the specificity of the hemolytic reaction for the specific antigen-antibody system, the next step was to determine whether or not the responsible antibody species was limited to the $\gamma_2$ complement-fixing cytotoxic antibody described by White (4) and by Benacerraf and coworkers(7). The standard anti-Ea antiserum shown in the upper graph of Fig. 1 was completely separated on a DEAE-cellulose ion-exchange column by way of the 2-step gradient into a $\gamma_2$ fraction and a $\gamma_1$ fraction. After adjustment of each fraction back to the initial volume of the serum placed on the column, they were analyzed for PCA(8) and hemagglutination titers. All the PCA activity was found to reside in the $\gamma_1$ fraction. Immunoelectrophoresis of the fractions with development by specific antigen confirmed the separation of these two 7S gamma globulins.

In the middle graph of Fig. 1, the difference in hemolytic capacity of the two fractions is shown. The $\gamma_2$ fraction accounts for all the hemolytic activity of the whole serum while the $\gamma_1$ fraction contains no activity. Thus the specificity for type of antibody was confirmed. The sensitivity of the hemolytic reaction for detecting the $\gamma_2$ antibody *under these conditions* was compared with the hemagglutination titer of the separated $\gamma_2$ fraction. Illustrative results are shown in Table II. Subsequent experiments not shown indicate that use of more complement in the reaction mixture increases the sensitivity of the assay.

Guinea pig isophilic and rabbit anti-Forssman macroglobulin antibody have been shown to be hemolytic, and Jackson and Landy(26) have found hemolysis of endotoxin-coated erythrocytes a sensitive assay for macroglobulin antiendotoxin antibody. Sera taken 9 days after immunization of guinea pigs with ovalbumin in complete Freund's adjuvant was fractionated in TCB on Sephadex G-200(20, 27). The small amounts of $\gamma_1$ antibody present at that time appeared in the second peak of the elution curve. The first peak contained non-precipitating antibody which hemagglutinated antigen-coupled erythrocytes at a dilution of 1:640 and failed to show anaphylactic activity by PCA test. This peak contained components which appeared as macroglobulins on disc electrophoresis and analytical ultracentrifugation. This fraction was analyzed for hemolytic activity under the conditions described above and, like the $\gamma_1$, showed no hemolytic activity. It was therefore of interest to examine the effect of having coupled antigen to the erythrocyte surface on the titer of a known anti-Forssman macroglobulin hemolysin. Ovalbumin-coupled sheep erythrocytes were reacted with a rabbit hemolysin which (against normal sheep red cells) had a titer of 8900 50% units per ml. With the conjugated cells, the same antiserum had a titer of 8910 50% units per ml.

The reproducibility of the hemolysin assay was investigated by repeated titrations of a single antiserum over a 7-month period. Freshly prepared dilutions of the antiserum and frozen previously prepared dilutions were run against 23 consecutive separately prepared lots of ovalbumin-coupled erythrocytes. The mean titer of these 23 determinations was 1124 50% units per ml with a standard deviation of 166 (= 14.8%). The angle of the individual plots was measured with a protractor: the mean slope was $-14.3°$ with a standard deviation of $\pm$ 3.8°.

*Discussion.* In attempting to adapt the bis-diazotized-benzidine method to the measure-

ment of hemolytic antibody, other workers have encountered serious problems with spontaneous hemolysis and lack of satisfactory reproducibility. The reason for the spontaneous hemolysis of the coupled cells is obscure but the problem disappears with the use of isotonic salt solutions having high buffering capacity, as the introduction of 0.11 M phosphate buffer by Gordon, Rose and Sehon clearly demonstrated for the case of the hemagglutination system. A major difference between the work presented here and previous attempts at adapting for passive immune hemolysis erythrocytes conjugated to protein antigen with BDB lies in the choice of the buffer systems in which the cells are prepared and reacted with antibody and complement. By selecting a univalent, non-anticomplementary buffer and adapting quantitative techniques to the preparation and distribution of the coupled cells, it has been possible to devise a reproducible hemolysin titration method. Since these cells do not remain intact when left overnight in Tris complement buffer but do in phosphate buffer, further improvement of reproducibility might follow adaptation of phosphate buffer (with appropriate adjustments of $Ca^{++}$ and $Mg^{++}$ ion concentrations) to the system.

The bulk of our experimentation has been done with complement held at 4 50% units per reaction volume. In a limited number of instances either more or less has been used, resulting in significant differences in titers in the directions one would expect. In the data shown in Table II where $\gamma_2$ has been separated from the antiserum, a lower sensitivity of the hemolytic reaction comparative to hemagglutination is apparent. However, even at this level it remains considerably more sensitive than specific precipitation with antigen in immunoelectrophoresis. Use of increased amounts of complement in the reaction may obliterate the discrepancy between hemolysin and hemagglutination titers. We would stress that under the conditions described, accurate comparison between results obtained on different days for various antisera depends upon careful replication of the complement concentration each time the test is performed.

The experiments with the $\gamma_1$ and $\gamma_2$ fractions of the guinea pig immunoglobulins support the results obtained by Benacerraf and coworkers(7) with tanned cells bearing antigen in passive immune hemolysis. The failure of the macroglobulin anti-Ea antibody to hemolyze antigen-coupled cells in our hands was a surprise in view of the knowledge of the capacity of macroglobulin isophilic antibody to lyse sheep erythrocytes. In parallel to the 7S system, it may be that the use of complete Freund's adjuvant in sensitization evokes a different species of immunomacroglobulin since the standard rabbit anti-Forssman macroglobulin antibody retained undiminished its ability to lyse antigen-coupled cells. At least it indicates that not all the Forssman antigen sites were blocked in the process of the conjugation of protein to red cell and that the fragility of the treated cells in the presence of the anti-Forssman macroglobulin antibody was neither diminished nor enhanced. The function of guinea pig immunomacroglobulin in the case of protein antigens thus remains obscure.

While it appears from the foregoing that not all the natural antigenic groupings on the coupled erythrocytes are blocked, covered or reacted by the conjugation process, there is no information about the number of protein antigen molecules which this mechanism successfully places on a reacted erythrocyte. As the process is chemical and involves conjugation mainly to aromatic amino acids, it is not surprising that despite their disparity in molecular weight, on a milligram weight basis the amounts of ovalbumin and bovine gamma globulin for an optimal protein:BDB ratio are identical(28). Consequently, direct comparison of hemolysin titers between antisera of different antigenic specificity is of questionable validity.

Theoretically, almost any soluble protein antigen could be coupled with bis-diazotized benzidine to erythrocytes. There are a few recognized problems in adapting the method to a new antigen, such as the presence of reactive competitors in the antigen solution, as in the case of glycine in Stavitsky's initial experiments with diphtheria toxoid(13). The results of many experiments suggest that the

ideal conditions for creation of a stable end product are approached when the concentration of antigen in the mixture is sufficient to favor spontaneous adsorption on the normal cells. At this point enough BDB is added to couple red cell to antigen without being in such excess that the red cells themselves are complexed. Hemagglutination in the macro-method, with specially designed round bottom test tubes,‡ in our experience remains superior to microtiter methods for screening the resultant cells for successful coupling of antigen and resistance to spontaneous hemolysis.

In practice, the already successful adaptation of the BDB coupling technique to insulin, pollen extracts, diphtheria toxoid, basic protein encephalitogen, and various gamma globulins and albumins for purposes of hemagglutination should be easily translatable into hemolysin assays. Studies with these and other protein-containing antigens, such as transplantation and experimental autoimmune disease antigens, provide a means of examining the role of cytotoxic antibody in immunopathology. The successful application of protein-carrying indicator cells to *in vitro* techniques such as Jerne plates would broaden the range of antigen-antibody systems available to study well beyond the current restrictions to natural red cell antigens and certain polysaccharides.

*Summary.* By modification of reagents and conduct of reaction, the well-known technique for coupling protein antigens to erythrocytes with bis-diazotized-benzidine, already useful in detecting specific antibody by hemagglutination, can be adapted to quantitative titration of hemolytic antibody by passive immune hemolysis. This paper describes the details of preparation of the indicator cells and shows the specificity of their reaction. When antisera against a protein antigen are prepared in guinea pigs, hemolysis in this system will occur only in the presence of the complement-fixing, cytotoxic, hemolytic $7S\gamma_2$ antibody. The reproducibility of the method described was determined by 23 consecutive titrations of a standard hemolytic antiserum: the mean value was 1124 50% hemolysin units with a standard deviation of ± 166 (= 14.8%). The sensitivity of the present technique was found to be 1/10th to 1/20th as sensitive as hemagglutination upon comparative analysis of isolated $\gamma_2$ antibody. This is an improvement over the sensitivity of current immunoelectrophoretic assays for the $\gamma_2$ antibody.

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‡ Kontes Glass Co., Vineland, N. J., 13 x 75 mm hemagglutination tubes (K89675).

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## Enzymatic Differences Between Carrot Taproot Secondary Phloem And Tissue Cultures Derived from Secondary Phloem.* (30819)

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Secondary phloem of the carrot taproot is highly differentiated and does not, under ordinary circumstances, give rise to any other tissue type. When however this tissue is aseptically excised from the root and maintained under tissue culture conditions, autogrowth occurs of dedifferentiated tissue which resembles carrot tumors(1,7). This dedifferentiated tissue can redifferentiate under proper cultural conditions and form a complete carrot(1,2).

The results reported here comprise the first part of a study designed to identify key enzymatic changes during dedifferentiation and redifferentiation.

*Methods.* All carrots (*Daucus carota*) used in these studies had healthy green shoots and unbroken taproots which ranged from 8-9 inches in length and 1-1½ inches in diameter.

Cultures initiated from secondary phloem were made by excising sections 2.0 × 3.5 mm cut as discs from the middle third of taproots which had been sterilized with a 5% (w/v) solution of sodium hypochlorite. These discs were planted on the surface of agar-solidified Heller's medium(3), modified by the addition of sucrose (3.0%), 2,4-dichlorophenoxyacetic acid (0.01 mμg%), and coconut milk (15.0 ml/100 ml). Cultures were incubated at 22-28°C under continuous illumination from warm-white fluorescent tubes.

TABLE I. Enzymatic Activity of Carrot Taproot Secondary Phloem and Cultured Tissue Derived from Secondary Phloem.

| Tissue | Avg act units/mg protein | Std dev | p |
|---|---|---|---|
| Peroxidase | | | |
| taproot | .0476 | .00367 | <.001 |
| culture | .445 | .300 | |
| Malic dehydrogenase | | | |
| taproot | .370 | .0565 | <.001 |
| culture | .00135 | .00147 | |
| Acid phosphatase | | | |
| taproot | .0198 | .00422 | <.2 but >.1 |
| culture | .0652 | .104 | |

Enzymatic activity of filtrates prepared from normal secondary phloem and from new growth only from primary explants of secondary phloem discs grown in culture were compared. Standard methods(4) were used for assay of the following enzymes: acid phosphatase, alkaline phosphatase, glyceraldehyde-3-phosphate dehydrogenase, malic dehydrogenase, glucose-6-phosphate dehydrogenase, peroxidase, and aldolase.

*Results and discussion.* Only peroxidase, acid phosphatase, and malic dehydrogenase were present in either the cultured material or taproot phloem (Table I). There are significant quantitative differences between malic dehydrogenase and peroxidase activities of cultured material and taproot phloem (Table I): the cultured material contains more per-

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