is evident that the Waddell and Butler(1) principle for determining intracellular pH cannot be applied to determination of the erythrocyte pH. The latter principle assumes that the pK′ for DMO in plasma and erythrocytes is equal. Experiments are in progress to test the correctness of this assumption.

*Summary.* *In vitro* experiments on rat blood to which DMO was added indicated that the $DMO^-$ becomes distributed between the plasma and the erythrocytes in a manner similar to a Donnan distribution. It was concluded that DMO cannot serve as a test substance for determination of the erythrocyte pH employing the principle proposed by Waddell and Butler.

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Received December 3, 1963. P.S.E.B.M., 1964, v116.

## Hemagglutination by Rheumatoid Factor of Cells Coated with Animal Gamma Globulins.* (29313)

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The rheumatoid factor is a gamma globulin, usually of high molecular weight (19S), found in the sera of most patients with rheumatoid arthritis and of some patients with other diseases(1). It is characterized by its unique reactivity with low molecular weight (7S) human gamma globulin (HGG) and rabbit gamma globulin (RGG). It has been suggested that the rheumatoid factor may represent an "autoantibody" with primary specificity for 7S HGG and with cross-reactivity with RGG(2,3). If rheumatoid factor is an antibody to 7S HGG with cross-reactivity with RGG, it might be expected to cross-react with gamma globulins of other species, analogous to the cross-reactivity of classical antibodies to HGG with gamma globulins of other species. The present study was therefore undertaken to investigate the reactivity of rheumatoid factor with gamma globulins of species other than human.

The bis-diazotized benzidine (BDB) hemagglutination technique(4) was found to be more satisfactory than the tanned sheep cell method(5) for use in this study. Using the BDB method, it has been demonstrated that rheumatoid sera react with the gamma globulins of several mammalian species and that this reactivity can be completely absorbed from rheumatoid sera with a preparation of insoluble HGG. The reactivity of rheumatoid factor with these mammalian gamma globulins is consistent with the hypothesis that rheumatoid factor is an antibody to HGG with cross-reactivity with rabbit, bovine, equine and other mammalian gamma globulins.

*Materials and methods.* *Sera.* Sera were obtained at intervals over a 2-year period from patients with classical rheumatoid arthritis. The sera were stored at —20°C. Prior to use in the tanned sheep cell hemagglutination system, sera were inactivated at 56°C for 30 minutes and absorbed with washed sheep erythrocytes. Inactivation and absorption were not necessary in the BDB hemagglutination system but were sometimes carried out for convenience in the comparison of results obtained with the tanned cell sys-

* Supported by U.S.P.H.S. grant.

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tem; this did not affect the results in the BDB system.

*Euglobulin solutions.* Euglobulins were prepared at 4°C by addition of 1 volume of serum to 19 volumes of distilled water. After standing 2-4 hours in the cold, they were centrifuged at 4°C for one hour at 2000 rpm. The euglobulin precipitate was dissolved in a solution of 0.075 M NaCl and 0.075 M phosphate at pH 7.3 (hereinafter referred to as pH 7.3 phosphate buffered saline).

*Proteins.* Human Cohn Fractions II (gamma globulin), IV-4 (alpha globulin), and V (albumin) from pooled human plasma were supplied by the Squibb Co. through the generosity of the American Red Cross. Bovine gamma globulin (BGG) was obtained from the Armour Co. Rabbit gamma globulin (RGG), equine gamma globulin (EqGG) and porcine gamma globulin (PGG) were obtained from Pentex, Inc. The animal gamma globulin preparations were shown by filter paper electrophoresis to have less than 5% of other serum proteins as impurities. The RGG preparations used were free of HGG as shown by their failure to react with rabbit anti-HGG serum either in agar diffusion analyses or in hemagglutination. Guinea pig gamma globulin (GpGG) and chicken gamma globulin (ChGG) were prepared from whole serum by starch block electrophoresis.

*Antisera.* The anti-HGG sera were prepared in rabbits by intravenous injection of alum-precipitated HGG. Prior to use these sera were absorbed with serum from humans with agammaglobulinemia (kindly supplied by Dr. Robert A. Good); after absorption, only one precipitin band was observed with HGG or human serum on Ouchterlony plates and in immunoelectrophoresis. The anti-RGG serum was prepared in a goat by repeated injection of RGG in complete Freund's adjuvant. Rabbit anti-BGG, anti-EqGG, anti-porcine globulin, anti-guinea pig globulin and anti-chicken globulin sera were obtained from Sylvana, Inc. The antisera to the animal globulins all exhibited multiple bands in agar diffusion analyses. All antisera were absorbed with human or sheep erythrocytes prior to use in hemagglutination experiments.

*Bis-diazotized benzidine solution.* Bis-diazotized benzidine (BDB) was prepared by addition at 0°C, of 175 mg sodium nitrite in 5 ml water to 230 mg benzidine dihydrochloride in 45 ml 0.2 N HCl. After 30 minutes, the BDB solution was either used immediately or quick-frozen in 0.5-1.0 ml aliquots in a dry ice-acetone bath and stored at −20°C for future use.

*BDB hemagglutination.* For coating of the cells, 0.1 ml of a 50% suspension of washed group "O" human erythrocytes was added to 3 ml of a solution of gamma globulin in pH 7.3 buffered saline containing 240 μg protein nitrogen per ml. A 1:30 to 1:50 solution of BDB was prepared by rapidly adding 0.5 ml of freshly thawed BDB solution to the appropriate volume of cold pH 7.3 phosphate buffered saline and mixing. Immediately, 1.0 ml of this dilute BDB solution was added, with mixing, to the red cell and antigen mixture. After standing for 15 minutes at room temperature with occasional gentle agitation, the cells were washed 3 times by centrifuging for 5 minutes at 1500 rpm at room temperature in 3.5 ml portions of diluent (a solution of 0.03% human Fraction IV-4 and 0.1% human Fraction V in pH 7.3 phosphate buffered saline). Some clumping of coated cells was usually encountered after the initial centrifugation during the washing procedure; when excessive, the use of a more dilute BDB solution usually averted this difficulty. After washing, the cells were suspended in 2.5 ml of the diluent and 0.05 ml of this suspension was added to 0.5 ml of serial dilutions of serum or euglobulin solution, also made in the diluent. The added cells were agitated gently to provide a homogenous suspension and were then allowed to settle at room temperature. Settling patterns were read at 3 or 18 hours, but were recorded only after the latter interval had elapsed. A button of cells in the center of the tube was read as negative. A loose ring of cells around a small central smooth mat was designated one-plus. A smooth mat of cells at the bottom of the tube without any ring around the edge was designated two-plus. Folding of the edges of the mat occurred in stronger reactions, but the use of higher designations was

not deemed useful. Reactions of two-plus (or greater) were interpreted as positive. Titers were recorded as the reciprocal of the highest dilution of serum (or of euglobulin solution corrected to original serum concentration) which yielded a positive agglutination pattern.

*Tanned sheep cell hemagglutination.* Washed sheep erythrocytes were treated with tannic acid at a concentration of 1:40,000. The tanned cells were then washed and coated with HGG, generally at a concentration of 240 μg protein N per ml(5). Tanned, "coated" cells were suspended in the same diluent used in the BDB hemagglutination method; rheumatoid sera and euglobulin solutions were also serially diluted in this diluent. Cells were added to serial dilutions of test sera and settling patterns were read and interpreted in a manner identical to that described for the BDB method.

*Hemagglutination inhibition.* The antigen to be used as an inhibitor was serially diluted using the diluent described above. To 0.25 ml of serial dilutions of inhibiting antigen was added 0.25 ml of a dilution of a euglobulin solution which contained 16 times the minimum quantity of rheumatoid factor necessary for a positive hemagglutination reaction. After incubating for 1 hour at room temperature, 0.05 ml of antigen-coated cells was added and allowed to settle as described above. The end point was read as the least amount of antigen capable of clearly inhibiting agglutination of the antigen-coated cells by the dilution of euglobulin solution used.

*"Aggregated" and "unaggregated" HGG.* HGG was subjected to sodium sulfate fractionation, according to the method of Christian(6). The first two fractions ($SS_1$ and $SS_2$) contained considerable unaggregated material, but, in addition, contained almost all of the soluble aggregates present in HGG. These fractions, relatively rich in aggregates, were referred to as "aggregated" HGG. The last 2 fractions ($SS_5$ and $SS_6$) were virtually free of aggregated HGG, but to insure further the complete absence of aggregates, they were subjected to centrifugation for 3½ to 4 hours in a Spincol Model L ultracentrifuge at 40,000 rpm. The supernatant from fractions $SS_5$ and $SS_6$ after such centrifugation was referred to as "unaggregated" HGG.

TABLE I. Hemagglutination Titers of Rheumatoid Factor (Euglobulin Solution from Serum Sm.) and Rabbit Antibody with Tanned Cells Coated with Unaggregated or Aggregated HGG.

| | Concentration of HGG used for coating (μg protein N per ml) | Rheumatoid euglobulin (Sm.) | Rabbit anti-HGG serum |
|---|---|---|---|
| Unaggregated HGG | 250 | 160 | 2560 |
| | 90 | 0* | 1280 |
| | 30 | 0 | 640 |
| | 10 | 0 | 160 |
| | 3 | 0 | 0 |
| Aggregated HGG | 192 | 2560 | 5120 |
| | 90 | 1280 | 10240 |
| | 30 | 1280 | 5120 |
| | 10 | 320 | 1280 |
| | 3 | 0 | 320 |

* 0 = negative at 1:20.

*Ultracentrifugal studies.* Analytical ultracentrifugation was performed in a Spinco Model E ultracentrifuge. Sucrose density gradient ultracentrifugation was performed in a Spinco Model L ultracentrifuge using a SW 39L rotor(7).

*Experimental and results.* Preliminary studies of the relative effectiveness of coating tanned sheep cells with aggregated and unaggregated gamma globulin were carried out, since previous observations(8-10) have suggested that the degree of coating of tanned sheep erythrocytes is dependent, at least in part, on the degree of denaturation and aggregation of the antigen used for coating. In Table I are shown the hemagglutination titers when tanned sheep cells, coated in unaggregated and aggregated HGG solutions of various concentrations, were added to serial dilutions of a rheumatoid euglobulin (Sm.) and rabbit anti-HGG. As Christian has shown (6), rheumatoid sera were relatively incapable of agglutinating tanned sheep cells coated with unaggregated 7S HGG, but agglutinated tanned cells in high titer if the cells were coated with aggregated 7S HGG. It was further noted, however, that rabbit anti-HGG also is less effective in agglutinating tanned cells coated with unaggregated HGG. The diminished effectiveness is best noted when the lower concentrations of HGG are used in coating (Table I).

TABLE II. Comparison of Hemagglutination Titers of Rheumatoid Whole Sera and Euglobulin Solutions with Cells Coated with Gamma Globulins by the BDB Method.

| Cells coated with | Serum preparation used | Rheumatoid arthritis serum used | | |
|---|---|---|---|---|
| | | Cl. | Eg. | Sm. |
| HGG | Serum | 0 | 0 | 1280 |
| | Euglobulin | 320 | 640 | 2560 |
| RGG | Serum | 40 | 40 | 10240 |
| | Euglobulin | 160 | 160 | 5120 |
| BGG | Serum | 160 | 160 | 2560 |
| | Euglobulin | 80 | 40 | 1280 |
| EqGG | Serum | 640 | 80 | 5120 |
| | Euglobulin | 640 | 160 | 2560 |

Unaggregated HGG thus seemed likely to be less effective than aggregated HGG in sensitizing tanned cells for hemagglutination reactions in general. A similar, but more marked, difference had been noted in this laboratory between unaggregated and aggregated hen ovalbumin in hemagglutination reactions with rabbit anti-ovalbumin sera(10). Nevertheless unaggregated ovalbumin was capable of coating cells effectively for agglutination by anti-ovalbumin if the BDB method was used. It was, therefore, anticipated that cells could be effectively coated with gamma globulins by the BDB method, relatively independently of whether the gamma globulins were aggregated or unaggregated.

Difficulties were encountered in the adaptation of the BDB hemagglutination system to the study of rheumatoid sera. When whole serum, known from prior studies to contain rheumatoid factor, was used in the BDB hemagglutination system, unexpectedly negative results were often encountered with cells coated with HGG. Rheumatoid sera Cl. and Eg., shown in Table II, were 2 such sera. However, as also shown in Table II, euglobulin solutions prepared from those sera yielded positive results with HGG-coated cells. Rheumatoid factor was readily detectable in some higher-titered rheumatoid sera (*e.g.*, Sm., Table II) whether whole serum or euglobulin preparations were used. In such instances, however, euglobulin solutions provided greater reproducibility in results. Prozones were sometimes encountered when whole rheumatoid sera were used, but were not observed with the euglobulin preparations.

Ultracentrifugal studies were performed to evaluate the nature of the proteins in the euglobulin preparations. Analytical ultracentrifugation of serum Sm. had revealed, in addition to the normal 7S and 19S peaks, a 22S peak such as is frequently found in rheumatoid sera(11). No other abnormal complexes were noted. In the euglobulin solutions, however, a heterogeneous array of soluble complexes was observed. As shown in Fig. 1, (upper pattern) many of the complexes have sedimentation coefficients greater than 19S. Intermediate (9-17S) complexes of the type described by Kunkel *et al*(12), were also observed. The complexes dissociated into fairly discrete 7S and 19S peaks in pH 3 glycine-HCl (Fig. 1, lower pattern). Sera Cl. and Eg. exhibited no detectable

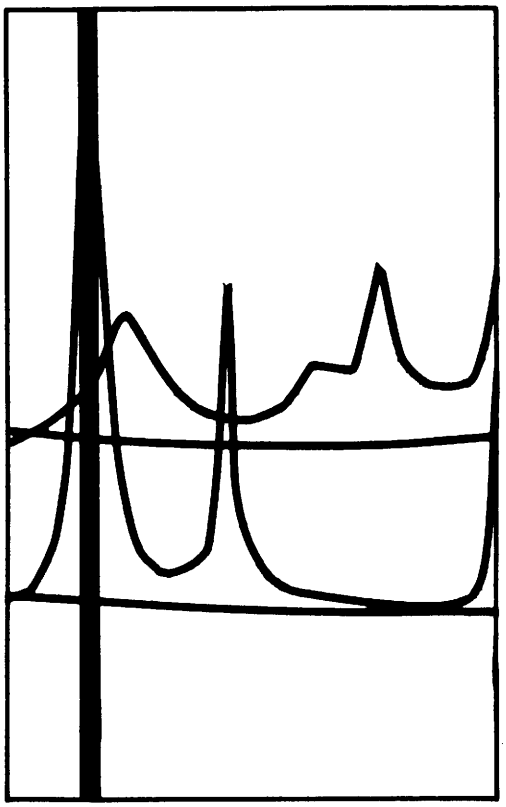

FIG. 1. Analytical ultracentrifugal patterns of rheumatoid euglobulin Sm. redissolved in pH 7.3 phosphate buffered saline (above) and in pH 3 glycine-HCl buffer (below). Reproduction of photograph taken 80 min after attaining speed of 39,460 rpm. Sedimentation proceeds from left to right.

TABLE III. Hemagglutination Titers of Rheumatoid Euglobulins and of Rabbit Antibody with Cells Coated by the BDB Method with Unaggregated or Aggregated HGG.

| | Concentration of HGG used for coating cells* (μg protein N/ml) | Sm. euglobulin | Cl. euglobulin | Rabbit anti-HGG serum |
|---|---|---|---|---|
| Unaggregated HGG | 200 | 1280 | 160 | >25600 |
| | 150 | 320 | 40 | 12800 |
| | 100 | 0† | 0 | 6400 |
| | 50 | 0 | 0 | 3200 |
| Aggregated HGG | 200 | >10240 | 640 | 12800 |
| | 150 | 1280 | 160 | 3200 |
| | 100 | 80 | 20 | 1600 |
| | 50 | 0 | 0 | 800 |

* Total protein in coating solution adjusted to 250 μg N/ml by addition of requisite amount of human serum albumin.

† 0 = negative at 1:20.

9-17S or 22S complexes on analytical ultracentrifugation. Density gradient ultracentrifugation of euglobulin fractions of these sera indicated that approximately 70% of the globulin was slow (7S) while 30% was rapid (19S) in sedimentation. It is probable that the greater effectiveness in hemagglutination studies of euglobulin in comparison with serum is a result of the relative enrichment of 19S over 7S globulins, thus providing a preparation with a diminished tendency for internal inhibition by 7S gamma globulins.

Problems were also encountered during these studies on the BDB hemagglutination system in the choice of a suitable diluent. Cells coated by BDB generally did not settle in saline or serum albumin buffers into a negative button in the bottoms of the tubes, thereby giving false positive patterns. The usual 1:500 solution of normal human serum used as diluent by many laboratories in rheumatoid agglutination reactions was unsatisfactory because the small amount of HGG in it was sufficient to inhibit rheumatoid factor activity. Similarly, 1:100 normal rabbit serum was found to inhibit agglutination of cells coated with RGG. The diluent which proved most satisfactory consisted of 0.1% human Fraction V and 0.03% human Fraction IV-4 in pH 7.3 phosphate buffered saline.

The BDB concentration (1:30 to 1:50) and gamma globulin concentration (240 μg protein nitrogen per ml) used represented optimal values determined in preliminary experiments with HGG and RGG. The BDB concentration used has proved optimal for numerous protein antigens studied in this and other laboratories.

The BDB hemagglutination system was compared with the tanned sheep cell hemagglutination system for detection of the rheumatoid factor. Washed human erythrocytes were coated by BDB with the same preparations of "aggregated" and "unaggregated" HGG which were used to coat tanned sheep erythrocytes in the experiment described in Table I. Cells coated by BDB with aggregated HGG were agglutinated by higher dilutions of rheumatoid factor than cells coated with unaggregated HGG (Table III), while the rabbit anti-HGG agglutinated cells coated with unaggregated HGG in higher titer than it did cells coated with aggregated HGG.

Human erythrocytes were coated by BDB with 7 different species of gamma globulins—HGG, RGG, BGG, EqGG, PGG, GpGG and ChGG. Rheumatoid euglobulin preparations reacted strongly with cells coated with HGG, RGG and EqGG (Table IV). They exhibited lesser reactivity with cells coated with BGG, PGG and GpGG, and little or no reactivity with cells coated with ChGG. Euglobulin preparations from normal control human sera produced little or no agglutination of cells coated with these gamma globulins.

Cells coated with RGG, BGG and EqGG were added to serial dilutions of whole rheumatoid sera. In contrast to the behavior of HGG-coated cells, these cells were agglutinated in comparable titers whether whole sera or euglobulin solutions were used (Table II). All hemagglutinating activity for cells

TABLE IV. BDB Hemagglutination Titers of Erythrocytes Coated with Different Gamma Globulins by Rheumatoid (R.A.) Euglobulin Solutions and by Rabbit Antisera to the Respective Globulins.

| | Gamma globulin used for coating | | | | | | |
|---|---|---|---|---|---|---|---|
| | HGG | RGG | BGG | EqGG | PGG | GpGG | ChGG |
| Sm. (R.A.) | 10240 | 10240 | 2560 | 2560 | 2560 | 1280 | 0 |
| Cl. (R.A.) | 1280 | 320 | 40 | 320 | 80 | 0 | 0 |
| Eg. (R.A.) | 1280 | 320 | 40 | 160 | 80 | 160 | 20 |
| Rabbit antiserum | 5120 | 5120* | — | 10240 | 160 | 10240 | 5120 |

* Goat anti-RGG used.

TABLE V. Hemagglutination Titers of Density Gradient Ultracentrifugal Fractions of Sm. Serum with Cells Coated by BDB with Various Gamma Globulins.

| Ultracentrifugal fraction number* | O.D.† (277 mμ) | Hemagglutination titer with cells coated with: | | | |
|---|---|---|---|---|---|
| | | HGG | RGG | BGG | EqGG |
| 1 | .075 | 0 | 0 | 0 | 0 |
| 2 | .100 | 0 | 0 | 0 | 0 |
| 3 | .372 | 0 | 0 | 0 | 0 |
| 4 | .680 | 0 | 0 | 0 | 0 |
| 5 | .790 | 0 | 0 | 0 | 0 |
| 6 | .478 | 0 | 0 | 0 | 0 |
| 7 | .208 | 200 | 100 | | 100 |
| 8 | .092 | 800 | 400 | 200 | 400 |
| 9 | .142 | 6400 | 6400 | 1600 | 3200 |

* Highest numbered fractions represent most rapidly sedimenting fractions.
† O.D. measured at 1:6 dilution of fraction.

coated with HGG, RGG, BGG and EqGG was found to be present in the euglobulin fractions of the rheumatoid sera. No hemagglutinating activity could be detected in the supernatants after euglobulin precipitation, although these supernatants still exhibited positive latex fixation reactions(13). It has been our experience that the latex test is less easily inhibited by 7S gamma globulins than are hemagglutination tests.

The hemagglutinating activity for the cells coated with the various animal gamma globulins was further characterized by subjecting serum Sm. to sucrose density gradient ultracentrifugation. The hemagglutinating activity for cells coated with RGG, BGG and EqGG was detected in the more rapidly sedimenting serum protein fractions, in association with the hemagglutinating activity for cells coated with HGG (Table V).

In Table VI are shown the minimal concentrations of HGG, RGG, BGG and EqGG required to inhibit rheumatoid agglutination of cells coated with each of the gamma globulins. HGG is extremely effective in inhibiting the agglutination of HGG-coated cells, concentrations of less than 1 μg protein nitrogen per ml sometimes being a very effective inhibitor. Much greater concentrations of HGG are required to inhibit rheumatoid euglobulin agglutination of cells coated with RGG, BGG and EqGG. With cells coated with the animal gamma globulins, the respective animal gamma globulin was always considerably more effective than the other gamma globulins in inhibition. It was possible, nevertheless, to remove all rheumatoid factor agglutinating activity (Table VII), not only that for HGG-coated cells, but also that for cells coated with RGG, BGG or EqGG, by absorbing these sera with a washed, insoluble preparation of BDB-treated HGG(14).

*Discussion.* By use of the BDB hemagglutination technique it has been possible to extend observations(2,3,5,7) on the reactivity of rheumatoid sera with the gamma globulins of various species. Not only were the rheumatoid sera reactive with HGG and RGG, but also with BGG, EqGG, PGG and GpGG; significant reactivity with ChGG was not demonstrated. The reactivity of rheumatoid sera with BGG and the lack of reactivity with

ChGG are in accord with reported observations of other workers, using different methods(15,16).

The strong reactivity of rheumatoid factor with EqGG was an unexpected finding in view of the observations of Vaughan(2) and of Edelman *et al*(7), that equine immune precipitates failed to absorb significant quantities of protein from rheumatoid sera or significantly to reduce hemagglutination titers with sensitized sheep cells. It is impossible to be certain that the sera studied by these earlier workers had a rheumatoid factor component reactive with EqGG, but if they did, as seems probable, the quantities so reacting may have been small enough to escape detection by the methods used. Alternatively, much of the antibody in the horse antigen-antibody precipitates used for absorption may have been $\beta_2$M or $\beta_2$A globulins, which would not provide a suitable absorbent for the rheumatoid factor; in our studies, and in accord with the observations of others, rheumatoid factor has not exhibited significant reactivity with immunoglobulins other than $\gamma_2$ (7S) globulins.

TABLE VI. Hemagglutination Inhibition.

| Inhibiting γ-globulin | Serum Sm. Cells coated with: HGG | RGG | BGG | EqGG | Serum Cl. Cells coated with: HGG | RGG | BGG | EqGG | Serum Eg. Cells coated with: HGG | RGG | BGG | EqGG |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| HGG | *1.25* | 25. | 25. | 100. | *<.1* | 100. | 50. | 50. | *1.6* | >100. | 6.2 | 6.2 |
| RGG | >100. | *6.2* | >100. | 100. | 1.6 | *25.* | >100. | >100. | >100. | *15.* | >100. | >100. |
| BGG | 50. | >100. | *12.5* | 100. | 100. | 100. | *6.2* | >100. | 100. | >100. | *<.5* | >100. |
| EqGG | >100. | >100. | >100. | *3.1* | 100. | — | >100. | *12.5* | >100. | — | >100. | *8.0* |

Concentrations (expressed in μg protein nitrogen per ml) of the 4 gamma globulins required to inhibit agglutination by 3 rheumatoid euglobulins of cells coated with each of these 4 gamma globulins. Cells were coated with gamma globulins by the BDB method. The amount of rheumatoid euglobulin used as agglutinator varied with each cell coating, being 16× the minimal amount required for agglutination in the absence of inhibitor.

TABLE VII. Hemagglutination Titers of Rheumatoid Euglobulin Preparations Before and After Absorption of the Sera with a Washed, Insoluble Preparation of Bis-Diazotized Benzidine-Treated HGG.

| Source of rheumatoid factor | Cells coated by BDB method with: | Hemagglutination titers Before absorption | After absorption |
|---|---|---|---|
| Sm. | HGG | 5120 | 0 |
| | RGG | 5120 | 20 |
| | BGG | 2560 | 0 |
| | EqGG | 2560 | 0 |
| Cl. | HGG | 640 | 0 |
| | RGG | 320 | 0 |
| | BGG | 40 | 0 |
| | EqGG | 320 | 0 |
| Eg. | HGG | 5120 | 0 |
| | RGG | 320 | 0 |
| | BGG | 40 | 0 |
| | EqGG | 160 | 0 |

The data also indicate that the components of rheumatoid arthritis sera which react with animal gamma globulins, like those reacting with HGG, sediment with the 19S gamma globulins. Moreover, all the components reactive with mammalian gamma globulins were absorbed from these sera by a preparation of insoluble human Fraction II, indicating that the rapidly sedimenting molecules which react with mammalian gamma globulins also react with HGG. On this basis, it seems proper to regard the components reactive with the other mammalian gamma globulins as a part of the rheumatoid factor. The limitations of hemagglutination methods are such that no precise quantitative conclusions can

be drawn from the data presented in the current study. Quantitative studies have, however, been performed(14) and provide evidence that only a relatively small portion of the total rheumatoid factor complex in each of these sera reacts with RGG or BGG.

No evidence has been obtained in this study to suggest that there are "rheumatoid factor" molecules capable of reacting with rabbit or other mammalian gamma globulins, but not with HGG. Reports by other workers suggesting the existence of such molecules have appeared(17,18); the gamma globulins used by these workers were probably less denatured than those used in our studies, and this would appear to be the most likely explanation for the differences in results.

The finding that HGG is not significantly more effective than RGG in inhibiting agglutination of RGG-coated cells is in accord with the observations of Edelman *et al*(7), in the sensitized sheep cell system and of Milgrom *et al*(17), studying the dispersion of alligator red cells sensitized with rabbit antibody to alligator erythrocytes. The finding that BGG and EqGG are more effective than HGG in inhibiting rheumatoid agglutination of BGG-coated and EqGG-coated cells, respectively, is in accord with the findings with RGG inhibition. It would seem probable that, as with RGG, the specific determinants in BGG and EqGG toward which rheumatoid factor specificity is directed, are present in greater abundance on BGG and EqGG, respectively, than on HGG.

In another study(14), BDB has been found capable of inducing aggregation of HGG, RGG and BGG; such aggregation must be assumed to represent, or to be accompanied by, denaturation. The present data do not permit a statement as to whether or not such denaturation occurs when gamma globulins are attached to the red cell surfaces by BDB, or whether such aggregation could enhance the reactivity of "unaggregated" gamma globulin used as the coating antigen. The degree of denaturation present in the gamma globulin *prior* to the coating step, however, has no bearing on the effectiveness of coating in the BDB system, while such prior aggregation is very important in the tanned cell system.

The greater effectiveness of coating tanned cells with HGG, when the HGG has been partially aggregated and denatured was first described by Christian in a study of hemagglutination by rheumatoid factor(6). Similar observations have been reported by others with other antigen-antibody systems. Rose and Witebsky(8) noted that heated thyroid extract was much more effective than the unheated extract in coating tanned cells for detection of anti-thyroid antibody. Bonilla-Soto *et al*(9) made similar observations with *Alternaria tenuis* antigen. George and Vaughan(10) studied urea-denatured ovalbumin, and found it to be much more effective than undenatured ovalbumin in coating tanned cells for detection of antibody. These observations have been extended in the present study to show that unaggregated HGG was less effective than aggregated HGG in coating tanned cells for detection of rabbit anti-HGG antibody, as well as of rheumatoid factor. Thus, the earlier observation(6) that, in rheumatoid hemagglutination, aggregated HGG is effective (while unaggregated HGG is not) does not provide definitive evidence that the rheumatoid factor has greater specificity for denatured than for undenatured HGG. Nor does the greater capacity of aggregated HGG for hemagglutination-inhibition or precipitability with rheumatoid factor (6,7) provide unequivocal evidence, for these greater reactivities of aggregated gamma globulins may merely be reflections of solubility differences between aggregated and unaggregated gamma globulins. On the other hand, we regard the slight, but significantly greater reactivity of cells coated by BDB with aggregated HGG in agglutinating with rheumatoid factor (Table III), particularly in view of their lesser agglutination with rabbit anti-HGG, as a more definite indication that rheumatoid factor may indeed have greater specificity for denatured gamma globulin.

*Summary and conclusions.* The bis-diazotized benzidine (BDB) hemagglutination system has been adapted to the study of the rheumatoid factor and has been demonstrated

to provide certain advantages over the tanned sheep cell hemagglutination method in the study of the rheumatoid factor. Euglobulin preparations from 3 rheumatoid arthritis sera have been shown by the BDB hemagglutination method to react with human, rabbit, bovine, equine, porcine and guinea pig gamma globulins; significant reactivity with chicken gamma globulin was not demonstrated. It has been shown that the reactivity with rabbit, bovine and equine gamma globulins is associated with rheumatoid factor activity both by euglobulin precipitation and ultracentrifugal fractionation. These reactivities with the animal gamma globulins were absorbed from rheumatoid sera by a preparation of insoluble HGG. The reactivities with the animal gamma globulins therefore are felt to represent a part of the rheumatoid factors of these sera. These findings are consistent with the hypothesis that the rheumatoid factor is antibody to human gamma globulin with cross-reactivity with the gamma globulins of other mammalian species.

We are grateful to Mrs. LeMoyne Smith, Mr. William Marchetti, Mr. Bradley Beach, and Mr. William Yoerger for technical assistance.

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Received February 4, 1964. P.S.E.B.M., 1964, v116.

## Quercetin Inhibition of Specific Histidine Decarboxylase. (29314)

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Control of histamine formation *in vivo* should be of value in medicine. One of the authors (GJM) investigated the bioflavonoids as histidine decarboxylase inhibitors in 1949(1). At that time, the studies were carried out using an enzyme prepared from kidney; today, it is known that the enzyme involved in histamine production *in vivo* differs from this. The enzyme in kidney extracts is primarily an aromatic L-amino acid decarboxylase with strong activity against 5-hydroxytryptophan and only a weak action on histidine; the enzyme obtained from mast cells (via mast cell tumors or a transplantable rat hepatoma) is a specific histidine decarboxylase, highly active against histidine and with no activity against 5-hydroxytryptophan or dihydroxyphenylalanine(2-5).

It seemed indicated to recheck the bioflavonoid molecules against the specific histidine decarboxylase, both *in vivo* and *in vitro*.

*Methods. Histidine decarboxylase activity.* Tissues (1 g each) homogenized in 5 ml cold phosphate buffer (0.1 M, pH 7.5 or 8.3)