THE ISOLATION AND BIOLOGICAL ACTIVITIES OF RABBIT γM- AND γG-ANTI-SALMONELLA TYPHIMURIUM ANTIBODIES

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Antibodies to the somatic antigens of Gram-negative bacilli have been demonstrated to occur in three classes of immunoglobulins (1–10). Identification of specific antibody has depended upon the association of immune activity with immunoglobulin-rich fractions prepared from whole serum by various physical and chemical techniques. Immune activity in these fractions has been measured by examining secondary properties of antigen-antibody interaction such as agglutination of particulate antigens or particulate carriers coated with antigen, preparation of live bacteria for phagocytosis, and complement-dependent bactericidal effect. The results of such experiments have led to the tentative conclusion that all or most of the immune proteins of the serum specific to the somatic antigens of Gram-negative bacilli are of the γM-class of immunoglobulins (3, 5, 8–10).

In the studies described below the secondary activities of highly purified rabbit γM- and γG-antibodies to the somatic antigens of *Salmonella typhimurium* were examined and were found to differ markedly from each other.

Methods and Materials

1. Preparation of the Antigen.—*Salmonella typhimurium*, strain 7[1] (4, 5, 12:z) *Salmonella paratyphi* B[2] (4, 5, 12:b), and *Escherichia coli* were grown in brain heart infusion broth (Difco Laboratories, Inc., Detroit) with constant agitation for 15 hours at 37°C. The "O" or somatic antigen was prepared as described (12). The heat-killed whole cell antigens used for experiments involving absorption and/or absorption-elution were utilized within 15 hours after preparation. Antigen preparations for assay of agglutination and for immunization were stored at 4°C without preservative.

2. Immunization.—Adult hybrid rabbits were injected with 1×10^{10} organisms of *S.*

typhimurium subcutaneously on day 1 and intravenously on days 3, 5, and 7. The animals were bled on day 16, 18, and 20 from the ear artery. The blood was allowed to clot, and was incubated first at 37°C for 1 hour and then for 15 hours at 4°C. The sera were separated by centrifugation, pooled, and stored aseptically at 4°C without added preservatives.

3. Fractionation of Serum.—In a typical experiment, 1.2 liters of serum were equilibrated by dialysis against 1000 volumes of 0.04 M potassium phosphate buffer, pH 8.0, at 4°C and poured through a DEAE-cellulose[3] column prepared so that 1 gm of dry weight of the anion exchanger was available for 2 ml of serum. The serum was passed through the anion exchanger and the 0.04 M, pH 8.0, buffer allowed to follow through until the OD of the effluent fell to 0.1 when measured at 280 mu in a Beckman DU spectrophotometer. The column was then treated with potassium phosphate buffer, 0.4 M, pH 8.0, which released most of the absorbed serum proteins from the anion exchanger. The effluents collected from the two buffer systems were pooled separately. To facilitate presentation of the data, the antibody prepared from the 0.04 M and 0.4 fractions will be identified as γG and γM respectively pending further description of their characteristics in this report.

4. Isolation of Antibody from the two Fractions.—The two fractions were dialyzed against phosphate-buffered saline (PBS) (0.15 M NaCl, 0.05 M sodium phosphate, pH 7.2), and the agglutinin titer was assayed. Specific adsorption of the antibodies was carried out by adding 1 volume of *S. typhimurium* suspension containing 1×10^{10} bacteria/ml in PBS to 1 volume of a dilution of DEAE fraction containing 50 agglutinating units/ml (see Methods and Materials, No. 6). The reaction mixture was stirred gently at 37°C for 90 minutes and at 4°C for 18 hours. Agglutinated bacteria were allowed to settle and the supernatant fluid was removed. The bacterial suspension was centrifuged at 30,000 g for 1 hour at 4°C, resuspended in PBS, and recentrifuged until the OD of supernatant fluid was less than 0.1 at 280 mμ in the Beckman DU spectrophotometer. Elution of the adsorbed globulin from the bacteria was accomplished by adding enough sodium acetate buffer, 0.1 M, pH 3.9, to obtain a volume equal to the initial volume of the serum fraction in the reaction mixture. The suspension was stirred at 37°C for 90 minutes and was then centrifuged at 30,000 g for 20 minutes at 37°C. The supernatant fluid was removed and concentrated by pressure dialysis against 0.1 M sodium phosphate buffer, pH 6.8 (13).

The antibody-containing solutions thus obtained by specific absorption and subsequent elution were further purified either by DEAE-cellulose anion exchange chromatography for the 0.04 M fraction (γG) (14) or gel filtration through G-200 sephadex[4] for the 0.4 M fraction (γM) (15). The antibody-rich fractions were concentrated by pressure dialysis, sterilized by passage through a cellulose acetate membrane,[5] (pore size 0.22 mμ), and stored at 4°C without preservative. This procedure was performed in three independent experiments utilizing sera collected from different groups of rabbits yielding three pairs of anti-*S. typhimurium* antibodies for comparison. The activities of these three pairs of isolated antibodies were stable for at least 4 months. In calculation of the specific activity and molarity of the antibody preparations the following molecular weights were assumed: (a) γM, 1,000,000; (b) 2-mercaptoethanol reduced subunits of γM, 160,000; and (c) γG, 160,000 (16, 17).

5. Characterization of Antibodies.—Immunoelectrophoresis was done by the modification of the microtechnique of Scheidegger (see reference 18). Specific antisera towards whole rabbit serum and to fragments of rabbit γG-globulin obtained by papain cleavage (19) were prepared in goats immunized with these antigens emulsified in Freund's adjuvants.

Protein concentration of the antibody solutions was measured by absorbance in a Beckman DU spectrophotometer in a 1.0 cm wide quartz cuvette assuming $E_{1\ per\ cent}^{280}$ to be 14.0 for the rabbit immunoglobulins (19) by a modification of Lowry's technique using rabbit γG-globulin

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[3] Schleicher and Schuell, Keene, New Hampshire, type 40, 0.87 meq/gram.

[4] Pharmacia, Upsala, Sweden.

[5] Millipore Corporation, Bedford, Massachusetts.

purified from DEAE-cellulose (14) as a standard (20). The $S_{20,w}$ of the antibody solution was determined by ultracentrifugation in a model E Beckman-Spinco analytical ultracentrifuge as described by Trautmann (21). The conditions of centrifugation were the following: (a) solvent; 0.1 M sodium phosphate, pH 6.8; (b) protein concentration; 2 to 4 mg per ml; and (c) rotor speed; 56,100 RPM at 20°C.

Ultracentrifugation in a sucrose gradient and analysis of the individual fractions for protein and antibody activity was done by methods described for immune proteins and whole serum (22).

Rabbit γG-antibodies to crystalline egg white lysozyme and albumin, diphtheria toxoid[6] (23), and *Salmonella typhimurium* endotoxin (Difco Laboratories, Inc.) (24) used as controls were isolated by established procedures and were further purified by chromatography on DEAE-cellulose (14).

To examine the effects of the reducing agent, 2-mercaptoethanol (2-ME) on antibody activity, the antibody and serum samples were treated according to the method of Chan and Deutsch (25) except that the solvent for the 2-ME and iodoacetamide was PBS.

6. Bacterial Agglutination.—Twofold dilutions of the preparation to be tested (0.25 ml) were mixed with an equal volume of heat-killed, washed bacterial suspension containing 1×10^9 microorganisms/ml in PBS. The mixtures were incubated at 37°C for 1 hour and overnight at 4°C. The titer of the sample is expressed as the reciprocal of the last dilution exhibiting activity, as judged by macroscopic settling pattern of the bacteria similar to the technique previously described for erythrocytes (3). For this purpose kimax, 31 x 100 mm, culture tubes with symmetrically rounded bases were most useful. For experiments involving antiglobulin reagents, an 0.5 ml aliquot of goat antiserum diluted with PBS was added to bacteria which had reacted with the antibody as described above and subsequently washed with PBS. The bacteria were resuspended in the diluted goat antiserum and incubated at 37°C for 1 hour and at 4°C for 15 hours. Control hyperimmune whole sera and normal sera were used throughout the experiments to assure the reactivity and stability of the antigen preparations. The specific agglutination activity of the individual antibodies was calculated by dividing the reciprocal of the titer by the protein concentration and is expressed as agglutination units/mg of antibody protein.

7. Complement-Dependent Bactericidal Assay.—The bactericidal activity of the antibody solutions for *S. typhimurium* in the presence of complement was assayed by the method of Muschel and Treffers (26). Three-tenths ml of a log phase bacterial culture (8.0 $\times$ 10^7 organisms/ml) was added to 1.3 ml of antibody dilution in saline containing 3.0 ml of precolostral calf serum, and 0.1 ml of a magnesium-saline diluent (Mg^{++} = 46.0 μg per ml). After incubation for 60 minutes at 37°C in a water-bath (kill period), 5.0 ml of BHI were added to each tube. The tubes were then reincubated for a 3 hour growth period, and the bacterial population was then measured by optical density at 650 mμ in a Bausch & Lomb "spectronic 20" spectrophotometer. The survival was determined by comparison to the optical density of the control tube, containing all components except antibody, equal to 100 per cent survival. The amount of antibody protein necessary to kill 50 per cent of the original inoculum was determined by interpolation or extrapolation from a probit (per cent survival) vs. log volume of the antibody-containing solution plot.

8. Preparation for Phagocytosis (26).—Female, Swiss, white mice of the 1CR strain, raised in a pathogen-free environment were obtained from Charles River Farms, Brookline, Massachusetts. *S. typhimurium* taken from an actively growing culture in brain heart infusion broth (Difco Laboratories, Inc.) were counted with a particle counter[7] and adjusted to a final

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concentration of 1.0×10^6 organisms/ml. The bacteria were mixed with an equal volume of the antibody preparation or appropriate control, and incubated with agitation at 4°C for 20 minutes. Aliquots of 0.2 ml of the reaction mixtures were injected intravenously in groups of mice. The animals were sacrificed 1 hour later, and the number of bacteria in the peripheral blood was counted by direct plating of serial dilutions of heart blood nutrient agar (Difco Laboratories, Inc.). Approximately 65 per cent of the injected bacteria were cleared from the circulation of control animals in 1 hour so that the end-point of the assay was taken as that amount of antibody which resulted in removal of 50 per cent of the remaining organisms in the circulation as compared to the control. Each value represents the data obtained from 8 to 16 mice.
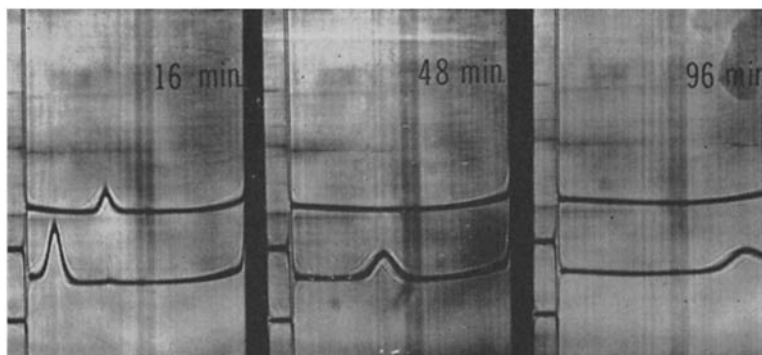

Fig. 1. Schlieren patterns of the rechromatographed antibodies eluted from the 0.4 M fraction (top) and 0.04 M fraction (bottom) of anti–*S. typhimurium* serum. The conditions of the experiment were: (*a*) protein concentrations, 2.4 mg/ml, top, and 4.0 mg/ml, bottom; (*b*) solvent 0.1 M sodium phosphate, pH 6.8; (*c*) rotor speed 56,100 RPM at 20°C; and (*d*) phase plate angle 60°C.

EXPERIMENTAL

Analysis of Antibody-Containing Solutions.—The degree of purity achieved in the preparation of antibody was determined utilizing various immunochemical techniques. Fig. 1 shows that each purified antibody solution contained a single homogeneous protein component during ultracentrifugation for 200 minutes at 56,100 RPM at 20°C. The calculated $S_{20,w}$ for the γM-fraction was 18.7S (top), and for the γG-fraction was 6.7S (bottom). Ultracentrifugation in a sucrose density gradient (Fig. 2) showed that antibody activity against *S. typhimurium*, as measured by bacterial agglutination, was located only in the major protein-containing fractions. The γM-antibody preparations contained some ultraviolet absorbing, serologically non-reactive material, which sedimented to the bottom of the gradient. The reduced and alkylated γM sedimented in the sucrose gradient with the characteristics of serum proteins of the 7S class. Coincident with this peak of protein was a definite low level of agglutination. The rapidly sedimenting material without immune activity observed in the un-

reduced preparations of γM did not have a detectable change of its ultracentrifugal characteristics in the sucrose gradient after treatment with 2-ME. Failure to visualize this rapidly sedimenting material by analytical ultracentrifugation probably was due to its low concentrations in the three preparations.

The specific somatic antigen of cells of *S. typhimurium* could absorb 89 to 95 per cent of the antibody protein (Table I). Cells of *S. paratyphi B* with similar somatic antigens (4, 5, 12) absorbed 70 to 81 per cent of the antibody protein. In contrast, *E. coli* cells with unrelated somatic antigens absorbed 5 to 7 per cent of the protein from the anti-*S. typhimurium* antibodies. This abosrption was apparently non-specific since similar amounts of protein were absorbed onto the three bacteria by rabbit antiprotein antibodies and serum
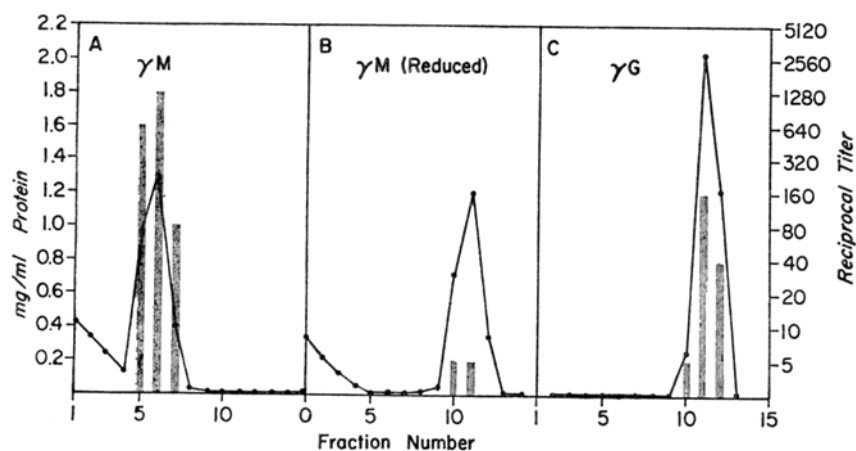

FIG. 2. The protein concentration and agglutination activity (see Methods and Materials No. 6) of fractions from sucrose density gradient ultracentrifugation of the rabbit immunoglobulins are shown.

albumin. Thus, approximately 85 per cent of the protein in the anti-*S. typhimurium* antibody preparations could be shown to react specifically with the antigen by this method. Treatment of the two classes of antibody preparations with 2-ME did not significantly change the amount of protein specifically absorbed by the bacteria. Antigen-binding properties of the reduced subunits of γM-antibodies have also been shown for erythrocyte, protein, and hapten antigens (25, 28–30).

Immunodiffusion studies using goat antiserum to rabbit serum proteins and to fragments of γG-globulin obtained by papain hydrolysis are summarized in Fig. 3. The γM-fraction contained a protein which showed fast gamma mobility by immunoelectrophoresis and shared identity with fragment I, but did not precipitate with antifragment III serum. By contrast, the antibody prepared from the (γG) fraction formed a single line of precipitation in the

slow gamma region by immunoelectrophoresis with all three antisera. The specificity of the antibodies is further shown by the common lines of precipitation formed with *S. typhimurium* endotoxin. These results are consistent with an immunochemical classification of these antibodies as γM- and γG-immunoglobulins.

TABLE I

Per Cent Protein Absorbed from Rabbit Serum Proteins by Salmonella typhimurium

Antibody	Concentration	Bacterial antigen		
		S. typhimurium	S. paratyphi B	E. Coli B-4
	mg/ml			
γM No. 1	0.055	92.2	72.2	5.3
γM No. 1 (2-ME)	0.051	90.4	63.8	4.6
γM No. 2	0.104	94.7	70.2	2.8
γM No. 2 (2-ME)	0.100	94.9	61.5	4.1
γM No. 3	0.115	95.6	75.0	3.5
γM No. 3 (2-ME)	0.109	91.5	65.8	4.1
γG No. 1	0.113	94.7	73.8	4.1
γG No. 2	0.098	94.3	74.9	3.8
γG No. 3	0.105	89.3	62.4	4.1
γG No. 3 (2-ME)	0.103	91.7	62.0	3.5
γG-antiendotoxin	0.111	93.5	62.0	4.8
γG-antilysozyme	0.138	5.8	6.2	5.7
γG-antidiphtheriztoxin	0.098	7.2	4.4	3.8
γM (normal)	0.143	7.0	6.4	5.8
γM (2-ME) (normal)	0.140	6.2	6.8	3.1
γG (normal)	0.120	7.3	7.0	5.7
Albumin	0.105	6.0	6.0	6.1

1.0 ml aliquots of specific anti-*S. typhimurium* antibody or antibody with specificity toward other antigens, on normal rabbit γM, γG, or serum albumin were incubated with an equal volume of bacteria $(1 \times 10^{10}$ organisms/ml) for 1 hour at 37°C. The tubes were allowed to stand in the cold at 4°C for 24 hours with occasional agitation and then centrifuged at 2400 g for 1 hour at 4°C. The protein in the supernate was measured by recording the absorbance at 280 mμ and the per cent of protein absorbed by the bacteria after this procedure is shown.

Comparison of the Specific Activity of the Two Classes of Rabbit Anti–S. Typhimurium Immunoglobulins.—

Bacterial agglutination: Agglutinating activity of the γM- and γG-anti–*S. typhimurium* antibodies prepared in three different experiments was compared. As shown in Table II, distinctive differences in the ability of these two classes of antibody to induce macroscopic agglutination were noted: (*a*) the threshold of sensitivity for detection of γM-anti–*S. typhimurium* by macroscopic agglutination ranged from 1.0 to 5.0 μg antibody protein/ml. By comparison the minimum requirement of γG-antibody to detect agglutination varied from

25.0 to 50.0 μg protein/ml; and (b) The specific activity of the anti–S. typhi-murium immunoglobulins, calculated as described in the methods and materials section, differed markedly. The three γM-preparations were calculated to
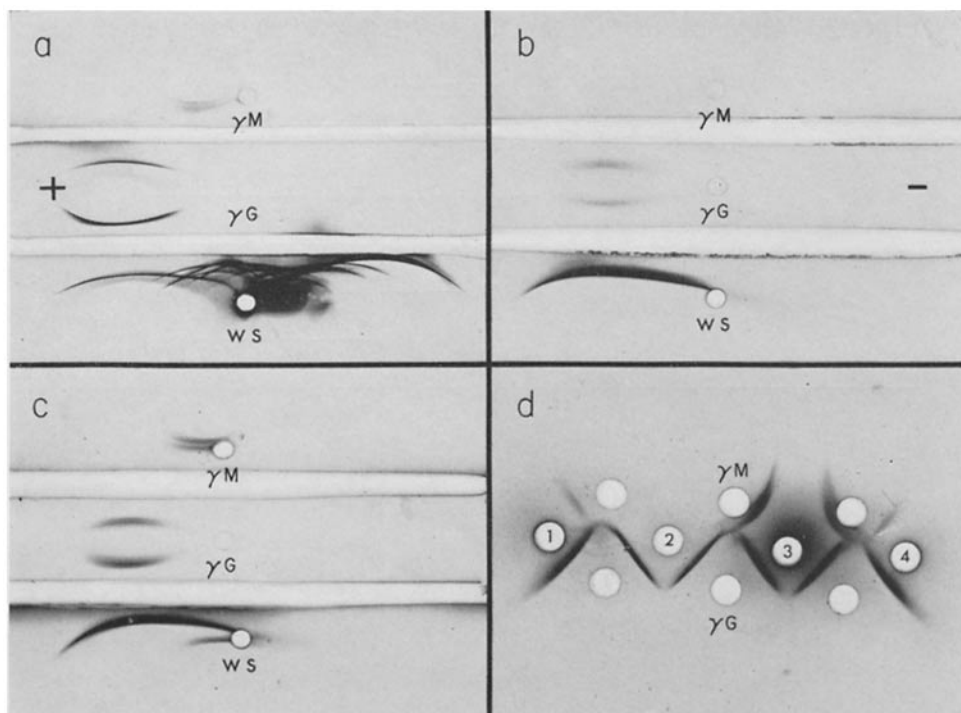

FIG. 3 a to 3 d. Immunodiffusion analyses of rabbit γM- and γG-anti–S. typhimurium antibodies. Figs. 3 a, 3 b, and 3 c are immunoelectrophoretic studies of the rabbit antibodies using 3 antisera. Following electrophoresis of γM (top well), γG (middle well), and rabbit hyperimmune anti–S. typhimurium "O" serum (bottom well), the precipitin arcs were developed with: (a) goat anti-rabbit serum proteins; (b) goat anti-rabbit γ-G fragment III; and (c) goat anti-rabbit immunoglobulins. Whole serum, WS.

Fig. 3 d contains γM-antibody in the top 3 wells and γG-antibody in the bottom 3 wells. To develop the precipitin lines the following reagents were placed in the middle line of wells: 1, goat anti-rabbit fragment I; 2, goat anti-rabbit γG-fragment III; 3, goat anti-rabbit immunoglobulins; and 4, Salmonella typhimurium endotoxin (Difco Laboratories, Inc.) 1.0 mg/ml.

have a mean specific activity of 645 agglutination units/mg (range 600 to 715) as contrasted to a mean of 72 agglutination units/mg (range 91 to 300) for the γG. To account for the difference in the molecular weight of these two antibodies, the specific activity of the γM may be multiplied by a factor of 6 so that the ratio of agglutinating activity of γM to γG is approximately

22. No prozone phenomenon involving simple macroscopic agglutination at high protein concentration was observed with either antibody.

Consistent with reports of other assays which measure agglutination of particulate carriers, the agglutinating activity of γM-globulin antibody was considerably reduced after treatment with 2-ME (24, 27, 28) while the activity

TABLE II

Specific Agglutination Activity of Rabbit Anti-Salmonella Typhimunum Antibodies

Antibody	Concentration	Antigen used for agglutination					S. paratyphi B
		S. typhimurium					
		Saline	Antiglobulin				saline
			Anti-RI	Antifragment III			
				0.2	0.1	0.02	
	mg/ml			*mg/ml*	*mg/ml*	*mg/ml*	
γM No. 1	0.05	32	1280	—	—	—	5
γM No. 1-2-ME	0.05	0	320	—	—	—	—
γM No. 2	0.88	640	40960	2560	640	640	80
γM No. 2-2-ME	0.88	40	20480	80	40	40	—
γM No. 3	0.54	320	20480	1280	320	320	40
γM No. 3-2-ME	0.54	16	10240	64	16	16	—
γG No. 1	0.88	80	640	2560	1280	640	10
γG No. 1-2-ME	0.88	80	640	2560	640	640	—
γG No. 2	1.05	320	1280	10240	5120	320	20
γG No. 2-2-ME	1.05	320	1280	20480	10240	320	—
γG No. 3	1.267	160	640	2560	640	160	20
γG No. 3-ME	1.267	160	640	2560	640	160	—
γG-Antiendotoxin	0.54	20	80	160	80	40	5

0.25 ml of twofold dilutions of anti–*S. typhimurium* immunoglobulins were incubated with an equal volume of heat-killed *S. typhimurium* (1×10^9 organisms/ml) suspended in PBS for 60 minutes at 37°C and overnight at 4°C with occasional shaking. The titer of agglutination was taken as the reciprocal of the last dilution, yielding an immune settling of the bacteria. The controls included normal rabbit γM- and γG-globulins. Rabbit antiprotein antibodies and normal goat serum showed no specific agglutination of *S. typhimurium* or enhancement of agglutination.

of the γG-globulin was unaffected. Further, Schrohenloher, *et al.* (30) reported that the reduced γM maintained a low level of agglutinating activity. Corollary to the decrease in specific activity as a result of 2-ME treatment was an increase in the minimal concentration of γM-antibody (50 to 100 μg/ml) required to induce detectable agglutination. The threshold of reduced γM was therefore approximately equal to the minimal level of γG-antibody necessary to produce agglutination.

The use of antiglobulin reagents has been shown to be a valuable tool for enhancing bacterial agglutination (31, 32). Optimum proportions of goat anti-rabbit γ-globulin serum (Table II) enhanced the agglutination by both antibodies. The increased titer ranged from 6 twofold dilutions for the γM-anti–S. typhimurium antibody, and 2 twofold dilutions for the γG-globulin. Addition of this antiglobulin antiserum to bacteria incubated with 2-ME reduced γM-antibody yielded a titer which was only 1 or 2 tube dilutions less than the enhancement of the intact γM. Through the use of goat anti-rabbit immunoglobulin serum, the agglutinating activity of 5 to 10 μg/ml of reduced γM could be detected. These results parallel those reported by Chan and Deutsch (25) and Jacot-Guillarmod and Isliker (28) which showed that addition of an anti-γM-antiglobulin reagent to reduced γM-antierythrocyte agglutinins resulted in agglutination titers identical with the intact antiglobulin treated γM-antibody (25).

The ability of the goat anti-γG-fragment III antiserum in higher concentration (2.0 μg antibody/ml) to enhance the agglutination titer of the γM-preparation remains unexplained. At least two possibilities were considered. The γM-preparation may have been contaminated with trace amounts of γG-antibody. It is also possible that the goat antifragment III antiserum had a level of antibody lower than the 1 μg/ml, directed towards the polypeptide moiety shared by all the immunoglobulins which was not detectable by precipitation in agar gel. At a concentration of 1.0 μg/ml, this goat antifragment III reagent enhanced bacterial agglutination only when the bacteria were incubated with γG-anti–S. typhimurium antibody.

Comparison of some Physical Properties of Rabbit Anti–S. typhimurium Antibodies.—

Effect of pH on stability of rabbit anti–S. typhimurium immunoglobulin-antigen bond: Acid dissociation of bacterial antigen-antibody bonds is utilized as a step in purification of antibody (33, 23). Thus, it was of interest to compare the stability of the two immunoglobulin-antigen bonds at various hydrogen-ion concentrations.

Equimolar concentrations of γM (0.20 mg/ml) and γG (0.03 mg/ml) were incubated at 37°C with an equal volume of S. typhimurium (1 × 10^{10} organisms/ml) suspended in PBS. The tubes were incubated at 4°C for 15 hours with occasional gentle agitation and then centrifuged at 10,000 g for 30 minutes. The bacterial pellet was washed twice with PBS at 4°C and then resuspended in 0.1 M sodium acetate buffers of various hydrogen-ion concentrations. The protein eluted from bacteria suspended in PBS or rabbit γG-antilysozyme antibody for each buffer used in the experiment were the controls.

As shown in Fig. 4, at pH 3.7 approximately 50 per cent of γM-antibody was dissociated and released into solution; in contrast, only 28 per cent of the γG-globulin dissociated at this pH. Approximately a tenfold increase in [H$^+$] was necessary to dissociate 50 per cent of the γG-globulin from the bacteria

(pH, 2.8); at this pH approximately 75 per cent of the γM-antibody was dissociated. Treatment of the γM with 2-ME slightly decreased the stability of the reduced subunits to remain bound to the antigen in the range pH 4–5.

Effect of absorption with increasing amounts of antigen upon the relative concentrations of γM- and γG-anti-S. typhimurium antibodies in whole serum: As another measure of "avidity", the decline in titer of the agglutination of γM-
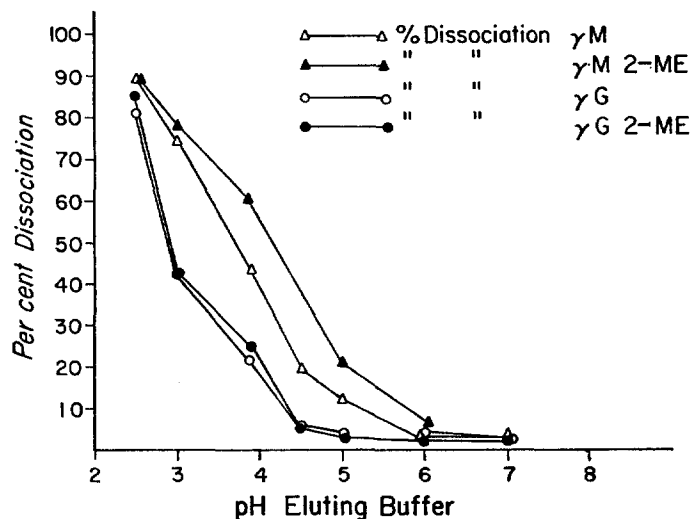

Fig. 4. Effect of hydrogen ion concentration upon the dissociation of rabbit anti-*S, typhi murium* immunoglobulin-antigen complexes. The relative stability of γM- and γG-antigen antibody bond was examined by measuring the release of antibody from specifically coated bacteria in solutions of increasing hydrogen concentration. The values obtained for each antibody at the various hydrogen ion concentrations are plotted as the per cent dissociated antibody (ordinate) *vs.* pH of the eluting buffer (abscissa).

and γG-anti-*S. typhimurium* antibodies in hyperimmune whole serum was measured following absorption with increasing concentrations of antigen.

As indicated in Fig. 5, at concentrations of 2×10^9 to $1.\times 10^{10}$ organisms/ml, virtually all the antibody extracted from whole serum is of the γG-class. Considering that the capacity for bacterial agglutination of γG-globulin antibody is lower than γM, the reduction in the titer after absorption with bacteria of γG-antibody is more significant. Only at a concentration of 2×10^{10} organisms per ml is there a significant reduction in the titer of γM observed after a single absorption.

Bactericidal activity: Table III shows the high order of difference between γM- and γG-anti-*S. typhimurium* antibodies testing for specific bactericidal activity. As little as 0.05 μg/ml protein of γM-anti-*S. typhimurium* antibody

was detectable with this assay in contrast to the minimal detectable value of 0.9 μg/ml protein of γG; *i.e.*, an eighteenfold difference. As was observed for bacterial agglutination, reduction with 2-ME did not completely ablate the
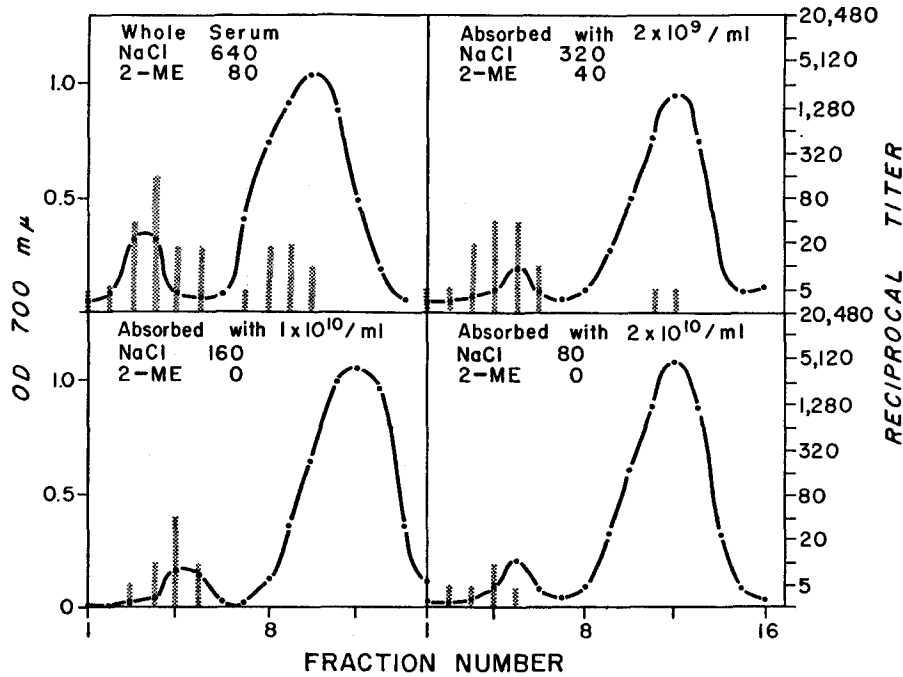

Fig. 5. Effect of absorption with increasing amounts of *S. typhimurium* somatic ("O") antigen upon the immunoglobulin content of hyperimmune serum. 0.5 ml aliquots of rabbit antiserum and various concentrations of *S. typhimurium*, suspended in PBS, were mixed, incubated at 37°C for 1 hour and at 4°C for 15 hours. The reaction mixture was centrifuged at 2000 g for 60 minutes at 4°C, and the supernates and the unabsorbed serum diluted 1:1 with PBS and were analyzed for antibody activity after ultracentrifugation in a sucrose gradient. Following centrifugation at 35,000 RPM at 4°C for 15 hours in a 39 SW rotor (beckman-spinco), 16 fractions were collected by piercing the bottom of the tube with a No. 22 needle (Becton, Dickinson and Co., Rutherford, New Jersey, 4.62 LNRS). The lines connecting the dots represent the protein concentration, and the agglutination titer of each fraction is shown. The agglutination titer of the whole serum and the serum treated with 2-ME are shown in the upper left of each diagram.

bactericidal activity of the γM-antibody. When concentrations were calculated on a molar basis, the activity of the 2-ME treated γM-antibody was found to be greater than that of γG-globulin; *i.e.*, a threefold difference. The threshold of detection for the reduced γM was 8.0 μg/ml protein. Exposure of the γG-anti-*S. typhimurium* antibody to 2-ME did not alter the capacity of this

TABLE III

Specific Bactericidal Activity of Rabbit Anti-Salmonella typhimurium Antibodies

Antibody	Molarity per 50 per cent kill	Mean
γM No. 1	3.24×10^{-11}	2.78×10^{-11}
γM No. 2	2.76×10^{-11}	
γM No. 3	2.34×10^{-11}	
γM No. 1-2-ME	1.67×10^{-7}	7.82×10^{-8}
γM No. 2-2-ME	2.92×10^{-8}	
γM No. 3-2-ME	3.84×10^{-8}	
γG No. 1	2.10×10^{-9}	2.17×10^{-8}
γG No. 2	3.10×10^{-9}	
γG No. 3	6.00×10^{-8}	
γG No. 1-2-ME	2.10×10^{-9}	2.17×10^{-8}
γG No. 2-2-ME	3.10×10^{-9}	
γG No. 3-2-ME	6.00×10^{-8}	
γG-Anti-S. typh.	1.10×10^{-9}	1.10×10^{-9}

Bacteriocidal activity was determined by the growth assay method of Muschel and Treffers (1956). The molarity of the antibody-containing solution inducing 50 per cent kill was taken as the end-point.

TABLE IV

Specific Enhancement of Clearance of Salmonella typhimurium in Mice by Anti-Salmonella typhimurium Antibodies

Antibody	Moles/50 per cent clearance
γM No. 1	1.0×10^{-15}*
γM No. 2	4.0×10^{-15}
γM No. 1-2-ME	2.35×10^{-14}
γM No. 2-2-ME	2.0×10^{-13}
γG No. 1	1.3×10^{-12}
γG No. 2	2.2×10^{-12}
γG No. 1-2-ME	2.7×10^{-12}
γG No. 2-2-ME	1.1×10^{-12}
γG-Anti-S. typhimurium endotoxin	

One volume of antibody was incubated with an equal volume of *S. typhimurium* taken from an exponentially growing culture containing 1.0×10^6 organisms/ml for 20 minutes at 4°C. 0.2 ml aliquots of the reaction mixture were injected into the tail veins, and the number of viable bacteria in the peripheral blood 1 hour later was counted by direct plating. The end-point was taken as that amount of antibody which permitted phagocytosis of 50 per cent of the total amount of injected organisms in the circulation as compared to the control animals.

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immunoglobulin to sensitize bacteria for lysis in a complement-dependent system.

Unique to the bacteria-complement system was a prozone at high concentrations of either antibody protein (1.0 mg/ml). Therefore, the prozone inhibition of bactericidal activity detected in whole serum could be ascribed to high concentration of immunoglobulins.

Opsonization: Consistent with the results recorded for bacterial agglutination and in the bactericidal assay, γM-globulin antibody showed greater ability to enhance *in vivo* clearance of *S. typhimurium* than the γG-antibody (Table IV). The lowest concentration of γM-globulin showing detectable opsonizing activity was 0.006 ± 0.003 μg/ml; 0.131 ± 0.100 μg/ml of the γG-preparation was necessary to obtain the same result. Opsonizing activity of γM-antibody was retained after reduction by 2-ME but at a somewhat reduced level so that threshold concentration for detection was 0.030 μg ± 0.100 μg/ml of protein. The activity of γG-antibody was unaffected by exposure to 2-ME.

DISCUSSION

Rabbit anti-*S. typhimurium* antibodies were isolated from two fractions of immune sera prepared by DEAE anion-exchange chromatography. The specifically absorbed antibody was dissociated from the bacteria by acid elution and repurified by anion-exchange chromatography and gel filtration. The two protein preparations were examined by several immunochemical criteria, with results which justified their identification as γM- and γG-globulins. Both antibodies, prepared in three independent experiments, contained less than 10 per cent non-antibody protein. The activity of the three pairs of anti-*S. typhimurium* immunoglobulins, by four different measures, was quite constant within the limits of experimental error. This suggests good reproducibility of the extraction procedure.

The identity of their immune specificity was based upon specific bacterial agglutination of *S. typhimurium* and precipitation of endotoxin from *S. typhimurium* in agar gel. However, the two kinds of antibody differed markedly in their secondary immune properties. The degree of difference between the two antibodies varied with the assay. γM was 22 times as active as the γG in inducing agglutination, 120 times as potent in sensitizing bacteria for complement-dependent kill, and 500 to 1000 times as efficient as the γG as an opsonin.

Such differences in secondary properties of γG- and γM-antibodies have been deomonstrated for a rabbit anti-sheep erythrocytes system (34–36). Using fractions of whole serum separated by ultracentrifugation and zone electrophoresis, antibodies of the γM-class sensitized erythrocytes for complement-dependent kill more efficiently than γG-globulins. Further, antibodies of the γM-class were shown to have a high intravascular degradation rate (t½, approximately 3.0 days) as compared to antibodies of the γG-class (approxi-

mately 8 to 10 days) (37), and a more rapid rate of dissociation from cellular antigens compared to the γG-globulin (38). The latter studies concluded that the relatively greater efficiency of the γM-antibodies to sensitize erythrocytes for complement-dependent lysis was due to the high rate of dissociation from erythrocyte-antibody complexes with transfer of intact immune activity to other erythrocytes. Our experiments, testing the complement-dependent bactericidal effect with purified antibodies, is consistent with this conclusion. Again, γG was the more "avid" immunoglobulin as measured by the greater stability of its complexes with S. typhimurium to solutions of increasing hydrogen-ion concentration and by its preferential binding to antigen compared to γM-antibody at high concentration of both immunoglobulins in whole hyperimmune serum. This inverse relationship between activity and "avidity" of γM and γG towards cellular antigens provoked the hypothesis that "avid" antibodies with a low rate of dissociation would be more efficient in neutralizing the biological activity of a protein antigen such as a bacterial toxin (38). In confirmation of this proposal, it was recently reported that the neutralization of diphtheria toxin was found predominantly in the γG group of rabbit and human immunoglobulins although both γM- and γG-antibodies to this protein were demonstrable by hemagglutination of sensitized erythrocytes (39). These experiments, however, utilized fractions of whole serum so the conclusion of this study, that γM-diphtheria antitoxin combines but does not neutralize diphtheria toxin, awaits further studies utilizing purified antibodies. Such studies are now in progress in this laboratory.

These two examples serve to extend the proposition that secondary immune activity of whole serum is related to properties of the individual immunoglobulin with which the antigen interacts. Many other differences between immunoglobulins of identical specificity have been identified as regards their secondary immune properties (39, 40, 7).

Consistent with previous reports (25, 28, 30), treatment of the γM-antibody with 2-ME results in the dissociation of the intact molecule to subunits which retain their immune and antigenic activity. Even at comparable molar concentrations of protein, 2-ME–treated γM-antibody showed less secondary immune activity than the intact molecule by all assays. As has been pointed out recently (30), reliance upon 2-ME activity as the sole criterium to distinguish immunoglobulin activity in whole serum must be critically reexamined.

Further demonstration of the combining property of the reduced subunits of the γM-antibody was shown by the use of antiglobulin reagents (25–28). Reagents with activity directed against γM enhanced the agglutination titer of bacteria which had combined both with the intact and reduced γM. However, full agglutinating activity of the reduced γM was not restored to the antiglobulin-enhanced titer of the intact γM.

Isolation of these two antibodies has permitted quantitation of the relation between the amount of protein and the observed immunological activity of the antibody. These data confirm the necessity of studying purified antibodies when the secondary effects of antigen-antibody interaction are measured. Since the specific activity of immunoglobulins may differ, direct relationships between the whole serum concentrations of antibody and immune activity in most antigen-antibody systems do not necessarily exist.

The use of specific antiglobulin reagents has offered a potential for resolving this problem. Analysis of sera containing "natural" or "immune" antibodies to the O antigen of *S. typhimurium* with the use of antiglobulin reagents has revealed that most of the antibody to the somatic antigen of *S. typhimurium* is of the γG-class of immunoglobulins (41). These findings challenge the concept that cellular or particulate antigens provoke primarily antibodies of the γM-class.

In the present studies, antibody of the γA-class immunoglobulins as described to occur in rabbit serum (42) was not isolated. The failure to isolate antibodies of this class may be related to observations made for the guinea pig that the immunoglobulin response following injection of *E. coli* consisted of predominantly γG-immunoglobulins (7). Thus, a low level of antibodies of the γA-class to *S. typhimurium* may have prevented extraction of this class of antibody. Another explanation may be surmised from the recent finding that an equine antibody (γ1, 11S antihapten), with high association constant did not precipitate with a hapten-carrier conjugate (43). Thus, other rabbit immunoglobulins may combine with *S. typhimurium* but possibly fail to induce agglutination or to dissociate from the antigen under conditions of this experiment. Studies are underway to isolate antibodies to other biologically active substances which may provoke sufficient antibodies of the γA-class to compare the biological activities of all rabbit immunoglobulins with identical immune specificity.

SUMMARY

Rabbit γM- and γG-anti-*S. typhimurium* antibodies were isolated by combined immune specific and physical methods and some of their properties in immunological systems were measured. γM-Antibody was detectable at lower concentrations and revealed a higher specific activity than the γG-globulins. Indirect studies indicated that the γG-globulin was the more "avid" immunoglobulin. Treatment of the γM-globulin with the reducing agent 2-ME decreased but did not destroy the immune activity of the subunits. The results confirm the necessity of analysis of purified immunoglobulin antibodies to evaluate the significance of their biological properties secondary to their interaction with antigens.

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