BACTERICIDAL METHOD FOR THE MEASUREMENT IN NORMAL SERUM OF ANTIBODY TO GRAM-NEGATIVE BACTERIA

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ABSTRACT

LANDY, MAURICE (National Cancer Institute, Bethesda, Md.), J. GABRIEL MICHAEL, AND JAMES L. WHITBY. Bactericidal method for the measurement in normal serum of antibody to gram-negative bacteria. J. Bacteriol. **83**:631–640. 1962.—The bactericidal effect of normal serum on gram-negative bacteria was investigated with the objective of determining whether this antibody-complement reaction could be used for the measurement of antibody. The major variables affecting the outcome of this complement-dependent reaction were explored systematically to establish the test conditions in which specific antibody was the limiting factor. Among these were the use of an excess of absorbed human serum as a complement source, inoculum, reaction conditions, and precise enumeration of surviving bacteria. It was demonstrated that the test conditions could be controlled so as to provide a sensitive and objective assay for antibody to representative strains of various gram-negative genera.

Over the past half century an extensive literature has accumulated on the bactericidal action in vitro of normal serum on gram-negative bacteria (Wilson and Miles, 1955; Skarnes and Watson, 1957). Although it has been known for a long time that normal serum possesses bactericidal activity, and that this property is extensively distributed among mammalian species, the factors in serum responsible for this effect have been defined only recently (Skarnes and Watson, 1957). The controversy in preceding decades concerning the identity of the components in serum responsible for this lethal action has now been resolved; serum bactericidal activity is attributable to specific antibody and a complex of serum accessory factors designated as complement (Muschel, 1960).

Investigations which reaffirmed the specificity of the antibody requirement have, in addition, shown that the quantity of this component which suffices to restore bactericidal activity to absorbed serum is exceedingly small (Muschel and Treffers, 1956; Landy, Trapani, and Rosen, 1960). It was this minimal antibody requirement which suggested to us that the bactericidal system could be utilized for the detection and measurement of the small amounts of antibody present in normal serum.

This paper describes an assay in which the requirements for complement, bacterial inoculum, and reaction conditions have been ascertained and standardized, so that addition of normal serum completes the reaction to the extent that it supplies specific antibody. The test provides a simple, objective, and sensitive assay for antibody to members of the *Enterobacteriaceae.*

MATERIALS AND METHODS

Collection of serum. Blood specimens were obtained by venipuncture in human donors and larger animals and by cardiac puncture in small animals. Individual blood specimens were allowed to clot at room temperature, and the serum was separated by centrifugation. Blood from groups of ten or more mice was pooled to provide sufficient volumes of serum. A 3-liter pool of human serum employed as a complement source throughout this work was comprised of 200-ml from each of 15 donors. Sera were distributed in amounts of 5 ml or less and stored at −20 C in stoppered tubes or flame-sealed ampoules. Individual samples were thawed as required.

Complement content of serum. Serum specimens were tested for their capacity to hemolyze amboceptor-sensitized sheep erythrocytes before and after they were absorbed with suspensions of gram-negative bacteria.

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Immune sera. An immunochemically standardized antityphoid serum, prepared by immunization of rabbits with typhoid Boivin antigen adsorbed on alum, and an immunochemically calibrated antienteritidis serum, prepared by immunization of rabbits with heat-killed *Salmonella enteritidis*, were kindly provided by A. M. Staub and R. J. Trapani.

Bacterial cultures. S. typhosa 0901 was the international standard nonmotile "O" strain. *Escherichia coli* 0127:B8 was supplied by A. Abrams from the culture collection of the Walter Reed Army Institute of Research. *Shigella dysenteriae* (type 1) was provided by R. Wedgewood, Western Reserve University. Strains of *Proteus*, *Serratia*, *Pseudomonas*, and *Aerobacter* from a variety of sources were selected on the basis of their susceptibility to the lethal effect of normal human serum.

RESULTS

The bactericidal action of fresh normal serum on gram-negative bacteria is attributable to a system comprised of specific antibody, the recognized components of complement, and divalent cation. The essential findings of a previous study (Landy, Trapani, and Rosen, 1960), which detailed these requirements for *S. typhosa* 0901, are summarized in Table 1. The removal, inactivation, or complexing of any of the components of this system results in loss of bactericidal activity. When all other components were present in excess, as little as 0.002 μg of antibody N was sufficient to fully restore bactericidal activity to the system.

TABLE 1. *Components of normal serum required for bactericidal activity against Salmonella typhosa 0901*

Component omitted	Treatment of fresh human serum	Bactericidal activity
None	None	+
Specific antibody	Absorbed with *S. typhosa*	−
None	Absorbed with *S. typhosa*, typhoid antibody added	+
Unrelated antibody	Absorbed with *S. marcescens*	+
Calcium, magnesium	Chelated with EDTA	−
C′1	Dialyzed (R1)	−
C′2	Heated 30 min at 52 C (R2)	−
C′3	Zymosan, 37 C (R3)	−
C′4	Hydrazine (R4)	−
Properdin	Zymosan, 17 C (RP)	+
None	R3 + R4	+

TABLE 2. *Effect of added complement on the bactericidal activity of normal sera for Salmonella typhosa*

Serum	Bactericidal titer* against *S. typhosa*	
	Unsupplemented serum	Supplemented with absorbed human serum as a complement source
Human	1:20	1:200
Horse	1:10	1:320
Cow	<1:5	1:20
Rabbit	1:10	1:20
Guinea pig	1:5	1:20
Rat	1:10	1:10

* Results are expressed as the highest dilution of serum showing more than 50% killing of an inoculum of 10^6 bacteria.

Fresh serum from all mammals tested, except mice, possessed bactericidal activity in varying degree against gram-negative organisms. It is known that the magnitude of this bactericidal activity also varies with different animals. In the present work it was found that for a given test organism this variation among mammalian sera is not always due to the same limiting factor. Normal sera from various species differed appreciably in their bactericidal activity against *S. typhosa*, depending on whether the test was performed with or without added complement (Table 2). Either antibody or complement may be the limiting factor in the bactericidal activity of normal serum.

These findings form the basis on which an assay for antibacterial antibody in normal serum must be founded. It is apparent that for antibody alone to be the limiting factor all other essential components of this multiple system in serum must be present in excess. Accordingly, it was necessary to determine the requirements for factors in serum other than antibody, the age and number of bacteria introduced, the conditions of time and temperature which would facilitate interaction of serum and bacteria, and the most practical means of assessing the outcome of this

reaction. With this information, the quantity of antibacterial antibody present in normal serum could be measured. The sections which follow deal individually with these requirements.

Absorbing suspensions. Initially, acetone-killed and dried bacteria were used for absorption of antibody from human serum, but these proved inefficient for complete absorption with retention of complement activity. Subsequently, the same was found for bacteria killed with mercurials or by boiling. The most suitable suspensions tested were those killed by minimal heating.

The test strains were grown on tryptose agar in Roux bottles and harvested in saline after 24 hr of incubation at 37 C. The dense suspension obtained was filtered through gauze and then heated for 1 hr. The minimal effective temperature for each strain was employed: 56 C for *S. typhosa* and 60 C for *E. coli*. The cells were washed three times with saline at 4 C and made up to a 10% suspension. For absorption, the suspension was centrifuged, and the supernatant fluid was replaced with the appropriate quantity of serum to be absorbed. The organisms were resuspended in the serum, and the mixture was kept at 4 C overnight, after which the bacteria were removed by centrifugation.

Absorbed human serum. Serum from any of a number of mammals provides complement active in the bactericidal reaction. It was convenient, however, to use pooled normal human serum for this purpose. Since this serum is bactericidal, it was necessary to remove antibody under conditions which would retain undiminished activity of complement. In the early phases of the work, it was found that absorption at 4 C with washed, heat-killed bacteria did not completely remove bactericidal activity unless bacterial suspensions and serum were employed in approximately equal volumes. Under these conditions, the content of hemolytic complement was greatly reduced. When absorption was carried out with serum diluted 1:5 with saline, antibody was readily and consistently removed with much smaller quantities of absorbing suspension. Furthermore, the content of complement was not reduced appreciably. In such absorbed diluted serum, the complement activity could not be maintained even when stored at −20 C; accordingly, absorbed serum was freshly prepared for each test.

The results of an experiment in which diluted and undiluted human serum were absorbed in parallel with graded quantities of *S. typhosa* are given in Table 3. It is apparent that a greater quantity of bacterial suspension was required for absorption of typhoid antibody from undiluted serum than from serum diluted 1:5. Indeed, bacterial concentrations of less than 50% were incapable of completely removing antibody from whole serum. The loss of antibacterial activity against *E. coli*, a serologically unrelated species, indicated that either antibody or complement (or both) was removed. In contrast, relatively low concentrations of *S. typhosa* sufficed to remove specific antibody from diluted serum while at the same time retaining complement and antibody for *E. coli*, as evidenced by bactericidal activity for this organism. Consequently, to assure specific removal of antibody from human serum, absorption was routinely performed on serum diluted 1:5 with saline, employing the minimal amount of bacterial suspension previously found to remove antibody completely.

TABLE 3. *Effect of diluting human serum on the specific removal of antibody with retention of complement activity**

Conditions for absorption		Bactericidal activity for	
Human serum	Concn of *S. typhosa*	*S. typhosa*	*E. coli*
	%		
Undiluted	50	0	32
	25	48	62
	12.5	48	71
	6.25	48	61
	3.12	59	90
	1.56	74	110
	—	99	100
Diluted 1:5 with saline	50	0	60
	25	0	79
	12.5	9	81
	6.25	0	100
	3.12	30	94
	1.56	56	92
	—	94	98

* For absorption, washed bacterial suspensions and human serum were mixed and allowed to stand at 4 C overnight. For test of bactericidal activity, 0.4 ml of absorbed serum and 10^6 organisms of the test strain in 0.1 ml were mixed and incubated for 1 hr at 37 C. Results are expressed as percentages of the original activity remaining.

Further data relating to the nonspecific removal of antibody by high concentrations of absorbing bacteria are given in another report (Michael, Whitby, and Landy, 1962), in which these findings are amplified and discussed with regard to the long-standing controversy concerning the specificity of natural antibodies.

Test conditions for the interaction of bacteria and normal serum. A number of factors pertaining to the inoculum and serum influenced the outcome of their interaction and were therefore examined systematically.

1) Complement:—The requirement for serum accessory factors, such as complement, was determined by varying the amount of absorbed human serum added to the test serum (antibody) and the bacterial inoculum. Although the complement source can be diluted to a considerable degree and still support significant bactericidal activity, the amount required for maximal bactericidal effect is much greater (Table 4). Accordingly, to ensure that complement would not be limiting, 0.3 ml of a 1:5 dilution, which afforded a substantial excess, was employed routinely.

2) Bacterial inoculum:—Bacteria were maintained on tryptose agar slants at 4 C and transferred at monthly intervals. For the test, growth from an 18-hr subculture on tryptose agar was harvested in saline and standardized by nephelometry. To determine the inoculum size most suitable for detecting bactericidal activity, the number of bacteria in the reaction mixture was varied, by tenfold increments, over a range extending from 10^4 to 10^8 (Table 5). The bactericidal effect was similar for inocula of 10^5 to 10^7. Agar-grown organisms were found to be more suitable than those grown in broth. Similar results were obtained with antibody supplied as a 1:10,000 dilution of rabbit antityphoid serum. With an inoculum of 10^2 or 10^3 cells, absorbed serum often retained some bactericidal activity; whereas, for inocula of larger number, adequate absorption was readily achieved. However, when the inoculum exceeded a certain number (10^9), the requirements for antibody and for complement became excessive for the system. For these reasons, an inoculum of 10^6 cells was selected for use.

TABLE 4. *Determination of the amount of absorbed human serum required for the bactericidal system**

Dilution of complement (absorbed human serum) with test mouse serum	*S. typhosa* killed by complement + normal mouse serum
	%
1:5	95
1:10	94
1:15	73
1:20	49
1:25	34
1:30	34
1:5†	0

* To 0.1 ml of normal mouse serum diluted 1:10 was added the bacterial inoculum of 10^6 organisms in 0.1 ml, followed by 0.3 ml of the indicated dilution of absorbed human serum; incubation was for 1 hr at 37 C.

† Control (mouse serum omitted).

TABLE 5. *Effect of the size of the inoculum on the efficiency of the bactericidal system**

S. typhosa inoculum	Per cent of inoculum killed by the bactericidal system	
	Broth-grown organisms	Agar-grown organisms
10^8	50	75
10^7	60	90
10^6	90	94
10^5	80	92
10^4	80	50

* To 0.1 ml of normal mouse serum diluted 1:10 was added the bacterial inoculum in 0.1 ml, followed by 0.3 ml of absorbed human serum (complement); incubation was for 1 hr at 37 C.

3) Diluent:—It has been reported that for some bacterial strains saline is toxic or even bactericidal (Meynell, 1958). For this and other reasons, most workers have employed various buffers in their studies of the bactericidal reaction. In the present work, physiological saline, Veronal, or phosphate buffers gave similar results. It was therefore apparent that the test strains were stable in saline. Moreover, the concentration of serum present in the reaction mixtures (complement source and test serum) is at least 12%, an amount providing sufficient buffering capacity as well as essential divalent cation. Under these circumstances, saline was employed as the simplest diluent.

4) Time and temperature requirements:—To determine the rate at which the standard inoculum of *S. typhosa*, *E. coli*, and *S. dysenteriae* was killed by normal antibody and complement, the reaction mixtures of bacteria and serum were

incubated at 37 C. The results of such an experiment with *E. coli* are given in Table 6. At the intervals shown, samples were removed and the number of viable cells determined.

The temperature requirements for the reaction with *E. coli* were likewise ascertained with the same concentrations of bacteria and serum, interacted for 60 min at the temperatures shown in Table 7. The findings show that the conditions of 1 hr at 37 C are suitable for routine use. Essentially similar results were obtained with the typhoid and dysentery test strains.

Assessment of bactericidal action. The bactericidal activity of sera under test can be arrived at in two dimensions. In one, the reduction in bacterial inoculum at a given dilution provides an indication of potency. In the second, a suitable range of serum concentrations provides the data for assigning a value to the serum activity.

1) Dilution scheme for plating out reaction mixtures:—Immediately after incubation, reaction mixtures (0.5 ml) were diluted 1:10 by addition of saline. From this 1:10 dilution, further dilutions were made, i.e., two 1.0-log (1:10) dilutions and six 0.5-log (1:3.2) dilutions. The range of these dilutions is sufficient both for enumeration of the inoculum and for estimation of a broad range of bactericidal activity.

2) Plating and enumeration:—From each dilution of the reaction mixture, 0.5-ml samples were added to two cups in a plastic tray (Disposo-Trays, Model 96 CV, Linbro Chemical Co., Inc., New Haven, Conn.) previously sterilized by 30 min exposure to ultraviolet light. These trays are disposable clear flexible plastic sheets, 30 cm by 20 cm, with 96 cuplike depressions 20 mm in diameter and 10 mm deep. When all reaction mixture dilutions had been transferred, the cups were quickly filled with 2 ml of tryptose agar at 44 C by means of a Cornwall automatic syringe. The trays were placed in closed containers to prevent drying and incubated at 37 C overnight. The number of colonies in the cups was then counted by means of indirect illumination from an X-ray viewing box.

TABLE 6. *Rate of the bactericidal reaction**

Time allotted for interaction of serum and bacteria	Per cent of inoculum of *E. coli* killed
min	
0	6
10	41
20	58
30	81
60	92
120	83

* To 0.1 ml of normal mouse serum diluted 1:10 was added 3.2×10^6 *E. coli* in 0.1 ml, followed by 0.3 ml of absorbed human serum (complement); incubation was at 37 C for the indicated time interval.

TABLE 7. *Effect of incubation temperature on the bactericidal reaction**

Incubation temp	Per cent of inoculum of *E. coli* killed by antibody and complement
C	
4	0
17	0
27	68
37	94
44	96

* To 0.1 ml of normal mouse serum diluted 1:10 was added 10^6 *E. coli* in 0.1 ml, followed by 0.3 ml of absorbed human serum (complement); incubation was for 1 hr at the indicated temperature.

3) Expression of bactericidal activity:—The dilution scheme had been devised so that at least three cups in each series would contain a readily countable number of bacterial colonies. In practice it was found that the maximal number of colonies per cup which could be counted with acceptable accuracy was about 50. The colonies in such cups were counted and multiplied by the dilution factor. This value was then divided by the control value and expressed as a percentage of the inoculum.

4) Conduct of the test:—The procedure is illustrated by the titration of the capacity of normal mouse serum to restore bactericidal activity to human serum absorbed with *S. typhosa*. In setting up the test, the serum is serially diluted in saline and 0.1-ml samples of the dilutions are transferred to 10 by 100 mm tubes. The bacterial inoculum, in a volume of 0.1 ml, is then added and the reaction mixture is completed by the addition of 0.3 ml of the absorbed human serum prepared as previously described. Controls for the bacterial inoculum and the absorbed serum are included. The tubes are shaken and incubated in a 37 C water bath for 1 hr, after which they are removed and the reaction terminated by diluting each of the mixtures with 4.5 ml of saline. Thereafter, the

TABLE 8. *Protocol of a typical bactericidal titration of antibody for S. typhosa in normal mouse serum**

Interaction of serum and bacteria			Number of bacterial colonies in dilutions of reaction mixtures								Surviving bacteria
			Dilution								
			10^{-2}	10^{-3}	$10^{-3.5}$	10^{-4}	$10^{-4.5}$	10^{-5}	$10^{-5.5}$	10^{-6}	
			Cup number								
Test product	Reaction mixture	Amount	1	2	3	4	5	6	7	8	(Per cent of inoculum)
		ml									
Mouse serum	AS	0.3	TM	TM	13	9	1	0	0	0	4.8
1:12	B	0.1	TM	TM	20	3	2	0	0	0	
	MS 1:12	0.1									
Mouse serum	AS	0.3	TM	TM	TM	43	12	4	0	0	38.4
1:15	B	0.1	TM	TM	TM	48	19	2	1	0	
	MS 1:15	0.1									
Mouse serum	AS	0.3	TM	TM	TM	TM	20	4	1	0	66.6
1:18	B	0.1	TM	TM	TM	TM	35	5	2	0	
	MS 1:18	0.1									
Antibody control	AS	0.3	TM	TM	TM	TM	35	10	4	2	100
	B	0.1	TM	TM	TM	TM	35	12	3	0	
	S	0.1									
Inoculum control	B	0.1	TM	TM	TM	TM	40	16	3	2	112
	S	0.4	TM	TM	TM	TM	42	14	3	1	

* Key: AS = absorbed human serum; B = bacteria; MS = mouse serum; S = saline; TM = too many colonies to count.

procedure leading to determination of bactericidal action is as described previously.

To illustrate the over-all procedure, including calculations, the results of an experiment are recorded in Table 8. Each number in the table represents the bacterial colonies which had developed in one cup containing 0.5 ml of a dilution of the reaction mixture. It can be seen that, for the antibody control (absorbed serum), 70 colonies grew from 1 ml of a $10^{-4.5}$ dilution, 22 from the 10^{-5}, and 7 from the $10^{-5.5}$, a total of 99 colonies. From the mouse serum 1:12, 33 colonies grew from the $10^{-3.5}$ dilution, 12 from 10^{-4}, and 3 from $10^{-4.5}$, a total of 48 colonies in all. The method selected to express the latter count as a percentage of the absorbed serum control was simply to convert the total value of the second to a percentage of the first, as follows:

$$\frac{48}{99} \times \frac{100}{10} = 4.8\%$$

Similarly, the value for the percentage of surviving bacteria can be calculated for the other reaction mixtures; e.g., mouse serum 1:15 is:

$$\frac{128}{99} \times \frac{100}{10^{0.5}} = 38.4\%$$

The absorbed serum control rather than the inoculum control was used as the basis for all calculations. In a satisfactory test, the value for the former did not differ greatly from that for the latter.

The results obtained were in turn converted to probits with the use of a standard percentage response-probit table (Fisher and Yates, 1953) and plotted on probit-log paper as probits of bacterial survivors against the log of the serum dose. For comparing the potency of individual sera, the dose in ml which resulted in 50% survivors was read off the slope. In determining the 50% value by this method, the slope is extremely steep, so that a serum may be so active at one dilution that no probability value can be assigned; at a dilution twice this, there may be so little activity that for the opposite reason no value can be assigned. Thus, in practice, it was essential to make a preliminary titration, to determine the range, and a second more elaborate test to determine activity more precisely.

5) Evidence for the specificity of the contribution made by test serum:—With the use of absorbed normal human serum as a source of accessory factors, the normal serum of most mammalian species, including the mouse, restored

bactericidal activity for various *Enterobacteriaceae*. The finding that mouse serum, which is nonbactericidal, restored activity to absorbed human serum was evidence that antibody was the most likely contribution.

This assumption was verified in the following ways. Mouse serum, absorbed with the test organism, was no longer capable of restoring bactericidal activity for the homologous strain, but was still fully effective against serologically unrelated gram-negative species. Table 9 shows the results of an experiment in which normal mouse serum was absorbed with graded amounts of *S. typhosa* and *E. coli;* the absorbed serum, together with a complement source, was then tested for bactericidal activity against these two strains. It is evident that, at the appropriate concentration of absorbing suspension, bactericidal activity was removed for the homologous strain but not for the other. It is noted that at the highest concentrations of absorbing suspensions there occurred some nonspecific removal of activity (see also Table 3).

Further evidence of a similar nature is presented in Table 10. Mouse serum was absorbed with a single concentration of three bacterial strains. The only situations in which activity was removed were those in which the absorbing and test strains were the same. Tests with specific immune serum, in which 0.1 ml of dilutions of

TABLE 9. *Effect of the quantity of absorbing agent on specific removal of antibody from normal mouse serum**

Mouse serum absorbed		Bactericidal activity for	
Strain	Amount	*S. typhosa*	*E. coli*
	%		
S. typhosa	50	0	80
	10	0	99
	1	40	99
	0.1	70	99
E. coli	50	25	0
	10	38	0
	1	98	40
	0.1	99	40
	0.01	99	92

* To 0.1 ml of the absorbed mouse serum, diluted 1:10, was added the bacterial inoculum in 0.1 ml, followed by 0.3 ml of absorbed human serum (complement); incubation was for 1 hr at 37 C. Results are expressed as percentages of the original activity remaining.

TABLE 10. *Specific absorption of antibody from mouse serum**

Bacterial suspension (1% final concn)	Bactericidal activity for		
	S. typhosa	*E. coli*	*S. dysenteriae*
S. typhosa	5	93	—
E. coli	90	0	93
S. dysenteriae	—	98	5
None	96	99	90

* To 0.1 ml of a 1:10 dilution of the test mouse serum and 10^6 organisms of the test strain in 0.1 ml was added 0.3 ml of absorbed human serum (as complement source); incubation was for 1 hr at 37 C. Results are expressed as percentages of activity remaining after absorption.

the order of 1:10,000 fully restored bactericidal activity to the complement source, provided strong evidence that factors in serum other than antibody exerted no significant effect.

Additional evidence for specific reactivity of the test serum, such as would be contributed by antibody, was provided by experiments in which purified somatic antigens were added to the system. It was consistently found that as little as 1 μg of homologous endotoxin would suppress the bactericidal activity of normal mouse serum and complement, whereas antigenically unrelated endotoxins, even in amounts of 100 μg, were without effect. These findings are presented in detail in another report (Michael et al., 1962), where the whole question of the specificity of natural antibodies is considered as a separate subject.

6) Bacterial test strains:—Most of the data on the standardization of this procedure were obtained with the use of three strains, viz., *E. coli*, *S. dysenteriae*, and *S. typhosa*, and the protocols presented are drawn from this experience. To determine whether the findings were applicable to other gram-negative bacterial species, additional strains were examined for susceptibility to the bactericidal action of normal human serum. These included serotypes 026, 055, 0111, and 0126 of *E. coli*, and representatives of other bacterial genera, viz., *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Serratia marcescens*, and *Aerobacter aerogenes*. To verify that in these situations antibody and complement were the factors required for killing of the strains by human serum, each was tested as follows. Human serum was absorbed at 4 C with heat-killed cells of the organism under test and then supple-

mented with graded amounts of normal human or mouse serum. The bactericidal reaction was then carried out with the test organism as previously described. The absorbed serum displayed no bactericidal activity; supplementation with human serum restored bactericidal activity against the test organism. The serum titer varied considerably for the various strains. Mouse serum restored bactericidal activity for most but not all strains.

7) Performance and sensitivity of the procedure:—To obtain information on reproducibility, a pool of normal mouse serum and a rabbit antityphoid serum were examined on a number of occasions over a period of several months for bactericidal activity against *S. typhosa*. The amount of serum required to kill 50% of the inoculum was calculated for each individual test. For 19 determinations, the median ED_{50} for mouse serum was 0.006 ml, and the extremes were ± 0.0015 ml (± 25%). For six determinations, the median ED_{50} for the immune serum was 0.0000025 ml, and the extremes were ± 28%. Essentially similar results on the same pool of mouse serum were obtained with *E. coli* and *S. dysenteriae*, where the variation again was of the order of ± 25%.

To obtain further information on reproducibility, replicate samples of normal mouse serum were tested on the same day, employing identical reagents but individual serum dilutions and different operators. Under these circumstances, the variation among six tests was ± 15% for *S. typhosa* and ± 25% for *E. coli*. The availability of immunochemically calibrated rabbit antisera to *S. typhosa* and to *S. enteritidis*, which possess the same somatic factors, made it possible to determine the sensitivity of the procedure with precipitating antibody as the reference standard. These sera contained 220 and 130 μg antibody N per ml, respectively. Six tests on the antityphoid serum gave a mean ED_{50} of 0.0000025 ml, which is equal to 0.0005 μg of antibody N; three tests on the *S. enteritidis* serum gave a mean ED_{50} of 0.0000037 ml, equal to 0.0005 μg of antibody N. Agglutination tests using homologous bacterial suspensions gave end point titers of 1:800 and 1:480, respectively, attesting to the far greater sensitivity of the bactericidal system in the measurement of antibody.

DISCUSSION

Several methods are available for the quantitative determination of antibody in immune serum. The quantity of antibody detectable by different procedures varies greatly. Furthermore, the procedures differ considerably as regards advantages and applications; selection of the method is in accord with the particular needs of the investigator. However, in all the methods the range of measurement and the accuracy are to a large extent limited to the concentration of antibody present in immune serum. The concentration of natural antibody in normal serum is generally so low that the conventional methods can provide at best only qualitative information.

Normal serum from various mammals is bactericidal for gram-negative bacteria, even in the absence of demonstrable agglutinins. This bactericidal activity has frequently been titrated by progressive dilution to determine the smallest quantity of serum which effects a specified reduction in the bacterial inoculum (Rowley, 1956; Fischer, 1959). In such tests all the necessary components in serum are diluted simultaneously. Thus, the procedure actually measures the particular component that happens to be limiting. Such procedures are not reliable for measuring a specific component where it is necessary to insure that all other components are present in sufficient concentration. With an appropriate source of complement freed of antibody to the test organism, addition of normal mammalian serum will restore bactericidal activity to the extent that it supplies antibody. The antibody content of the test serum can then be titrated by adding progressively smaller quantities to constant volumes of complement and bacteria.

The quantity of antibody required for bactericidal action is extremely small. This suggested to us that the reaction could be utilized to detect and to measure the very low levels of antibody present in normal mammalian serum. Moreover, mouse serum, which possesses no inherent bactericidal action (Marcus, Esplin, and Donaldson, 1954), was capable of restoring bactericidal activity to the complement source just as any other mammalian serum tested. Hence, under appropriate conditions, the bactericidal reaction will serve for the assay of low-titered antibody in normal mammalian sera.

The present work describes a system where the following conditions of test have been provided: (i) serum-sensitive, antigenically-stable strains; (ii) a source of complement freed of antibody; (iii) inoculum of appropriate age and size; (iv) suitable conditions of time and temperature for interaction of serum and bacteria; and (v) a direct plating technique for enumeration of survivors.

Although these conditions have been dealt with individually in the experimental section, some are of such importance as to merit further comment. In selecting test cultures it should be noted that strains vary greatly in their susceptibility to the bactericidal action of normal serum. Of the many factors which contribute to this variability, only a few have been well defined. Perhaps the best understood are the capsular antigens, such as Vi and K, which interfere with the action of antibody against somatic antigen (Felix and Pitt, 1951). It is noteworthy that antibody to the capsular antigen, together with complement, can also function in the bactericidal reaction (Muschel, Chamberlin, and Osawa, 1958), but this kind of antibody is seldom found in normal serum (Landy and Lamb, 1953); antibody to somatic components is widespread (Gaines and Landy, 1955; Neter et al., 1955). Although most enteric species possess capsular components to some degree (Kauffmann, 1954), suitable strains of all genera have been found by direct testing for serum sensitivity.

Most investigators working on the bactericidal effect of normal serum have employed inocula ranging from 10^2 to 10^3 organisms. We found a proportional reduction in bactericidal count over a fairly wide range of bacterial concentrations. We selected an inoculum of 10^6 bacteria because it proved difficult to absorb natural antibody from the complement source, so as to render it nonbactericidal for smaller inocula. Secondly, survivors of an inoculum of 10^6 bacteria, even if they constitute a relatively small fraction of the input, can be counted with greater accuracy than would be the case with survivors of an inoculum of the order of 10^2 to 10^3.

From a practical standpoint, the most cumbersome aspect of this method is the determination of the outcome of the interaction between serum and bacteria. It is, of course, possible to determine the effects of antibody and complement on bacterial viability by methods other than plate count. Indirect means, such as photometric measurement of outgrowth of surviving bacteria (Muschel and Treffers, 1956) and reduction of neotetrazolium (Nagington, 1956), have been utilized to assess bactericidal action; there are, no doubt, still other indirect alternatives which could be used. The usefulness of the classical plating technique is self evident, but as generally performed, it is a time-consuming expensive procedure, especially when carried out on a rather large scale. In the present work, the utilization of disposable plastic trays, each of which serves as 96 plates in miniature, avoids this practical problem to a considerable extent.

Some of the data presented in this and other reports of different facets of our investigations, in which bactericidal antibody was shown to be present in sera devoid of agglutinating antibody, could be explained reasonably on the basis of the higher degree of sensitivity inherent in the bactericidal assay. There is, however, at least one other interpretation of these findings which should also be considered. Although natural antibodies possess the same specificities as immune antibodies, they may function in different ways, perhaps as a consequence of differences in their physical properties (Michael et al., 1962). It would be desirable to separate these antibodies from both normal and immune serum and to assess their functional capabilities in parallel tests.

The sensitivity of various methods for measurement of immune antibody has been reviewed and tabulated by Boyd (1956), and it is of interest to compare the bactericidal method with established procedures. In this comparison, specific precipitation is perhaps the least sensitive method. Bacterial agglutination, complement fixation, and toxin neutralization occupy an intermediate position, although some of the methods based on in vivo anaphylactic reactions are appreciably more sensitive. However, the only methods which compare in sensitivity with that described in this report are passive hemagglutination, using tanned erythrocytes coated with protein antigens, and tests based on the reaction of lytic antibody and complement. The bactericidal method would appear, therefore, to be among the most sensitive of the recognized procedures for the measurement of antibody.

Examples of the use and application of the bactericidal assay for natural antibody are not given in this report. The procedure has, however, been applied by us to the exploration of two areas of natural immunity: studies on the origin, specificity, and distribution of natural antibodies to *Enterobacteriaceae* (Michael et al., 1962), and alterations in the level of natural antibodies in mice after administration of endotoxin (Whitby et al., 1961). The levels of antibody to be dealt with in both situations were at the limit of sensitivity of older methods, but were well within the capacity of the bactericidal method to detect, and to measure with assurance, specific antibody in a variety of normal sera. Thus, with the procedure described in this communication, new information was obtained on important facets of natural immunity which had been difficult to resolve with previous methodology.

LITERATURE CITED

BOYD, W. C. 1956. Fundamentals of immunology. 3rd ed. Interscience Publishers Inc., New York.

FELIX, A., AND R. M. PITT. 1951. The pathogenic and immunogenic activities of *Salmonella typhi* in relation to its antigenic constituents. J. Hyg. **49**:92–110.

FISCHER, J. 1959. Zur Beeinflussung der Serumbakterizidie. Schweiz. Z. allgem. Pathol. u. Bakteriol. **22**:827–843.

FISHER, R. A., AND F. YATES. 1953. Statistical tables for biological, agricultural and medical research. Oliver and Boyd, Ltd., Edinburgh.

GAINES, S., AND M. LANDY. 1955. Prevalence of antibody to Pseudomonas in normal human sera. J. Bacteriol. **69**:628–633.

KAUFFMANN, F. 1954. Enterobacteriaceae. 2nd ed. Munksgaard, Copenhagen.

LANDY, M., AND E. LAMB. 1953. Estimation of Vi antibody employing erythrocytes treated with purified Vi antigen. Proc. Soc. Exptl. Biol. Med. **82**:593–598.

LANDY, M., R. J. TRAPANI, AND F. S. ROSEN. 1960. Inactivation of endotoxin by a humoral component. VI. Two separate systems required for viable and killed *Salmonella typhosa*. J. Clin. Invest. **39**:352–363.

MARCUS, S., D. W. ESPLIN, AND D. M. DONALDSON. 1954. Lack of bactericidal effect of mouse serum on a number of common microorganisms. Science **119**:877.

MEYNELL, G. B. 1958. The effect of sudden chilling on *Escherichia coli*. J. Gen. Microbiol. **19**:380–389.

MICHAEL, J. G., J. L. WHITBY, AND M. LANDY. 1962. Studies on natural antibodies to gram-negative bacteria. J. Exptl. Med. **115**:131–146.

MUSCHEL, L. H. 1960. Serum bactericidal actions. Ann. N. Y. Acad. Sci. **88**:1265-1272.

MUSCHEL, L. H., R. H. CHAMBERLIN, AND E. OSAWA. 1958. Bactericidal activity of normal serum against bacterial cultures. I. Activity against *Salmonella typhi* strains. Proc. Soc. Exptl. Biol. Med. **97**:376–382.

MUSCHEL, L. H., AND H. P. TREFFERS. 1956. Quantitative studies on the bactericidal actions of serum and complement. I. A rapid photometric growth assay for bactericidal activity. J. Immunol. **76**:1–10.

NAGINGTON, J. 1956. The bactericidal action of typhoid antibody. Brit. J. Exptl. Pathol. **37**: 385-396.

NETER, E., O. WESTPHAL, O. LUDERITZ, R. M. GINO, AND E. A. GORZYNSKI. 1955. Demonstration of antibodies against enteropathogenic *Escherichia coli* in sera of children of various ages. Pediatrics **16**:801–808.

ROWLEY, D. 1956. Rapidly induced changes in the level of nonspecific immunity in laboratory animals. Brit. J. Exptl. Pathol. **37**:223-234.

SKARNES, R. C., AND D. W. WATSON. 1957. Antimicrobial factors of normal tissues and fluids. Bacteriol. Rev. **21**:273–294.

WHITBY, J. L., J. G. MICHAEL, M. W. WOODS, AND M. LANDY. 1961. Possible mechanisms whereby endotoxins evoke increased nonspecific resistance to infection. Bacteriol. Rev. **25**:437–446.

WILSON, G. S., AND A. A. MILES. 1955. (Topley and Wilson) Principles of bacteriology and immunity. 4th ed. The Williams and Wilkins Co., Baltimore.