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Received May 4, 1963. P.S.E.B.M., 1963, v113.

A Simplified Method for Bactericidal Assay of Natural Antibodies Against Gram-negative Bacteria. (28513)

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It is now established that the bactericidal activity of fresh normal serum against Gram-negative bacteria is attributable to a system comprised of specific antibody, complement, and divalent cation(1,2). We have applied this knowledge to the measurement of natural antibodies in normal serum by using a complement source freed of antibody and appropriate sensitive strains of Gram-negative bacteria(3). The test serum restores bactericidal activity to the system to the extent that it contributes specific antibody. This assay has provided useful information as to the origin, specificity, and distribution of natural antibodies to Gram-negative bacteria (4) and the effects of endotoxin on the levels of natural antibodies in mice(5).

However, there are factors which tend to limit the general usefulness of this method (3), *viz.* (a) use of human serum as a complement source, which necessitates removal of natural antibody by absorption with suspensions of Gram-negative bacteria and (b) use of an inoculum of 10^6 bacteria which requires the plating out of an extended series of decimal dilutions of bacteria and serum to enumerate the survivors. This is time consuming and requires large numbers of pipettes and test tubes.

Modifications of the method have been devised to make the test more practical for general use and to increase its overall efficiency. Precolostral calf serum was employed as a complement source lacking in certain natural antibodies(6), and the bacterial inoculum was reduced to 10^2 cells. These changes, together with modifications in the performance of the assay, provided a rapid and simple method without any loss in precision.

Materials. Calf serum. Newborn calves of Holstein and Guernsey breeds were removed from their mothers prior to suckling and exsanguinated.* The blood was allowed to stand at room temperature for 1-3 hours to facilitate clotting, then kept at 4°C overnight. The serum was separated by centrifugation at 4°C and stored in rubber stoppered serum bottles at −50°C. Individual samples were thawed as needed; unused portions were discarded.

Rabbit serum. Adult New Zealand white rabbits were bled by cardiac puncture, and the serum from 18 rabbits was pooled and stored at −30°C.

Bacterial cultures. The various test strains of *Enterobacteriaceae,* susceptible to the bactericidal action of fresh human serum(3) were from the culture collection of the Walter Reed Army Institute of Research made available through the courtesy of Mr. A. Abrams: *Salmonella typhosa* 0901; *Shigella dysenteriae* (Type 1); *Escherichia coli* 0127 B:8; *Pseudomonas aerugenosa* (25-A-2); *Aerobacter aerogenes* (Type 1 NCTC #418); and *Klebsiella pneumoniae* K-12 (35-A-38). All were smooth cultures, not agglutinated by acriflavin or by the boiling of

* These sera were obtained for us from dairy herds in Maryland and Ohio by Mr. Robert Benson, Division Research Services, Nat. Inst. Health, and Dr. Robert Henthorne, Laboratory Animal Facilities, Ohio State Univ., Columbus, respectively. Sera obtained from herds in Colorado were purchased from Colorado Serum Co., Denver.

saline suspensions for one hour.

Methods. Preparation of inoculum. The various test strains of bacteria were grown at 37°C for 18 hours on brain heart infusion agar (Difco), except *S. typhosa* which was grown on nutrient agar. The cells were harvested in sterile normal saline and suspensions were standardized by means of a Klett-Summerson photoelectric colorimeter (filter #42) and a calibration curve to give a concentration of 200 viable cells per ml.

Assay procedure. Test sera and complement (calf serum) were thawed and diluted in chilled sterile saline. All reagents and glassware were kept in an ice bath during preparation of the reaction mixtures of complement, test serum and bacteria; the quantities employed were sufficient for subsequent tests in quintuplicate. Equal volumes of calf serum and dilutions of the serum under test were mixed, after which 2 volumes of the standardized inoculum were added. In the control, saline was substituted for the diluted test serum. Each of the reaction mixtures was dispersed with the aid of a Vortex Jr. Mixer and 1 ml aliquots were distributed into each of 5 sterile 25 mm × 200 mm test tubes with metal closures. Individual tubes then contained 0.25 ml of diluted test serum, 0.25 ml of calf serum and 100 viable cells in a total volume of 1 ml.

The tubes were incubated in a 37°C water bath for 1 hour, after which 8 ml of melted brain-heart infusion agar kept at approximately 45°C was added to each tube by means of a sterile Cornwall automatic syringe fitted with a 3 inch 15 gauge hypodermic needle. The tubes were swirled to assure mixing and slanted at an acute angle so as to yield the maximum surface area of agar. Once the agar had solidified the tubes were transferred to a 37°C incubator. After 18-24 hours incubation the colonies which grew out were counted and the percentage of surviving bacteria for each dilution of test serum was determined as follows:

$$\% \text{ survival} = \frac{\text{Total No. colonies in 5 tubes (test serum)}}{\text{Total No. colonies in 5 tubes (control)}} \times 100$$

TABLE I. Titration of Normal Rabbit Serum for Antibody Against *E. coli* 0127 B:8.

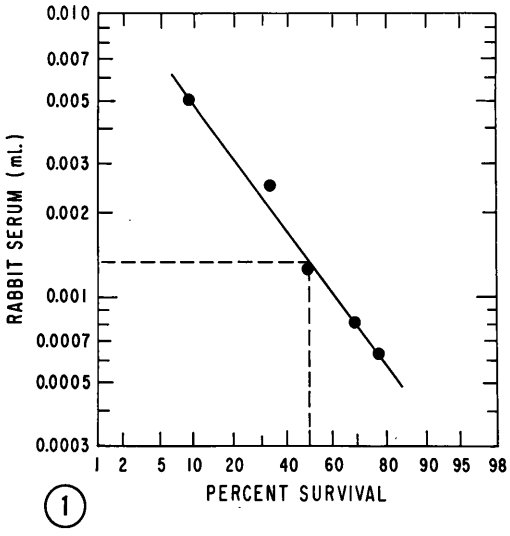
	No. of colonies in dilutions of serum					
	Dilutions					
Tube No.	1:50	1:100	1:200	1:300	1:400	Control
1	4	52	54	72	84	118
2	6	34	50	79	77	121
3	17	31	50	79	74	107
4	7	28	50	72	89	102
5	16	34	55	64	90	86
Total	50	179	259	366	414	534
% survival	9.4	33.5	48.5	68.5	77.5	100

Each tube contained 0.25 ml of diluted normal rabbit serum, 0.25 ml calf complement, and approximately 100 bacteria in a total volume of 1 ml. The control contained 0.25 ml of saline in the place of diluted rabbit serum.

The results were plotted on probability-logarithmic paper (K and E #358-24) and from the slope of serum dose *vs* percentage survivors was read the quantity of test serum (ml) which yielded 50% survivors; this value is hereafter referred to as the SD_{50}.

Results. A typical assay. Titration of the natural antibody against *E. coli* 0127 in pooled normal rabbit serum is depicted in the form of a protocol in Table I. The viable counts for quintuplicate samples of each serum dilution differ considerably. Whatever the magnitude of variation, all of the individual values were employed in each instance. Despite the evident diversity of these replicates, differences in the totals consistently corresponded to the dose increments of serum. A probability-logarithm plot of these data provided a slope of dose-response from which the SD_{50} for this particular serum was visually estimated as 0.00134 ml (Fig. 1). Experiments were performed to determine the effect of adding the reagents separately to the tubes as opposed to mixing them prior to addition; both procedures gave similar results. Accordingly, because of speed and ease in handling, admixture of all reagents prior to dispensing into tubes was the routine adopted.

Precolostral calf serum as a complement source. A natural source of complement could be used directly provided it was demonstrably free of natural antibodies to the test organism. Twenty batches of serum from new-



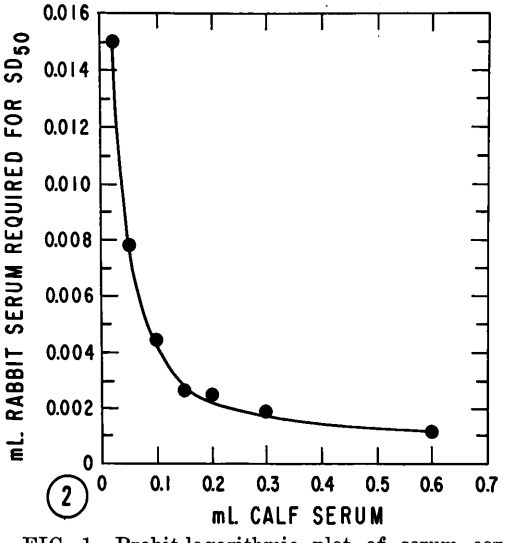


FIG. 1. Probit-logarithmic plot of serum concentration vs. percent survival.

FIG. 2. Effect of increasing amounts of calf complement on SD_{50} for normal rabbit serum, titrated against *E. coli*.

born calves were screened against the 6 test strains of Gram-negative bacteria. To each tube was added 0.25 ml of calf serum together with 100 viable cells of the test organism in a total volume of 1 ml. Calf serum from the same batch, heated at 56°C for ½ hour to inactivate complement, provided the control. All determinations were made in quintuplicate and carried out in the manner previously described. The results (Table II) show a consistent absence of bactericidal activity against certain strains, such as *E. coli* and *Pseud. aerugenosa;* activity was variable against other strains such as *S. typhosa,* being found in some sera but not in others; while all samples tested displayed bactericidal activity against *K. pneumoneae.*

To determine whether the observed bactericidal activity of calf serum involved specific antibody, a sample of Batch 7 was diluted with an equal volume of cold normal saline and aliquots of this diluted serum were absorbed overnight at 4°C with heat-killed cells of *S. typhosa* and *Shig. dysenteriae* respectively. Following removal of the bacterial cells, tests for residual bactericidal activity were made with several bacterial strains. The homologous strain was found to have removed activity whereas a heterologous strain had not, *i.e.,* the bactericidal activity of the calf serum was specific.†

Influence of calf serum concentration on bactericidal activity. To determine the amount of calf serum appropriate for use in the assay, pooled normal rabbit serum was titrated for antibody to *E. coli* while simultaneously varying the concentration of calf serum in the reaction mixture. The SD_{50} values thus obtained were plotted against the volumes of calf serum employed. This relationship is shown in Fig. 2; beyond the 0.2 ml level the increase in bactericidal activity with added calf serum was less pronounced. Thus when 0.2 ml of calf serum was utilized SD_{50} was 0.00250 ml; a 3-fold increase in quantity of calf serum reduced SD_{50} to 0.00125 ml. It would be desirable, on both theoretical and practical grounds, to supply a large excess of complement; however, the quantities of calf serum that would be in-

† It was noted that certain batches of calf serum which exhibited bactericidal activity for *Salm. typhosa* were not affected by absorption with the homologous organism. Moreover, the killing effect was observed only when the sera and bacteria were in contact at 4°C. The effect was directly related to time of incubation at this temperature. To obviate this complication, reagents, including *Salm. typhosa,* were kept at room temperature prior to admixture and incubation at 37°C.

TABLE II. Bactericidal Activity of Precolostral Calf Serum.

Test organism	Bactericidal activity* Calf No. 1	2	3	4	5	6	7	8	9	10
Salm. typhosa	3+	—	3+	—	2+	1+	2+	2+	1+	—
Shig. shiga	3+	—	2+	3+	2+	3+	3+	3+	—	—
A. aerogenes	—	1+	—	—	1+	—	1+	—	—	—
Kleb. pneumoniae		3+	3+	2+	3+	3+	3+	2+	2+	1+
Pseud. aerugenosa	—	—	—	—	—	—	—	—	—	—
E. coli	—	—	—	—	—	—	—	—	—	—

* 3+ 76–100% death
2+ 51– 75% "
1+ 26– 50% "
— 0– 25% "
Controls consisted of calf serum heated for ½ hour at 56°C.
Reaction mixture contained 0.25 ml of calf serum in a total volume of 1 ml.

volved made this impractical. In the titration of coli antibody in normal rabbit serum similar SD_{50} values were obtained when 0.25 ml amounts of calf serum from various batches were employed. Accordingly, it was decided that calf serum in the amount of 0.25 ml per reaction mixture represented an acceptable compromise and this increment was thereafter employed routinely. A precise estimate of complement activity is obtained by use of immune antibody in excess and decreasing increments of calf serum.

Bacterial inoculum. The effect of inoculum size on the sensitivity of the test was determined in experiments which initially utilized rabbit serum titrated against *E. coli.* Illustrative findings (Table III) show that as regards percentage of bacteria killed by natural antibody and complement, an inoculum of 10^2 is in the same range as that obtained with 10^6 bacteria. Moreover, tests with immune (rabbit) serum indicated that here too the percentages of cells killed in high and in low inocula were similar.

Reproducibility. The pool of normal rabbit serum was titrated on 15 separate occasions for antibody to *E. coli.* Sight estimates of the SD_{50}'s ranged from 0.00123 ml to 0.00205 ml; the mean value for these data was 0.00157 ml. As calculated by the method of least squares the mean SD_{50} was 0.00162 ml (0.00120 ml-0.00232 ml). The method of Fieller(7) was employed to calculate the 95% confidence interval for each titration. The distribution of these intervals is presented in Table IV. Eleven of the 15 confidence intervals were less than 0.0004 ml in length. Moreover, beyond the 4 to 5 dilutions routinely tested, an increase in number of serum dilutions employed in the assay did not make for a more precise estimate of the

TABLE IV. Distribution of 95% Confidence Intervals Calculated for Replicate Titrations of *E. coli* Antibody in Normal Rabbit Serum.

Magnitude of confidence interval (ml)	No. determinations within interval
.0001	1
.0002	8
.0003	2
.0004	0
.0005	1
.0006	0
.0011	1
.0024	1

TABLE III. Effect of Inoculum Size on Bactericidal Potency of Normal and Immune Antibody Against *E. coli* 0127B:8.

Serum	No. viables per reaction mixture	SD_{50} (ml of rabbit serum)	95% confidence interval
Normal	110	1.40×10^{-3}	$1.53 \times 10^{-3} - 1.28 \times 10^{-3}$
	1,200,000	1.98×10^{-3}	$2.28 \times 10^{-3} - 1.76 \times 10^{-3}$
Immune	86	8.03×10^{-6}	$8.57 \times 10^{-6} - 7.50 \times 10^{-6}$
	1,400,000	9.60×10^{-6}	$10.27 \times 10^{-6} - 8.92 \times 10^{-6}$

SD_{50}. Even in a titration involving 12 dilutions the length of the 95% confidence interval did not differ appreciably from that obtained in titrations involving only 4 dilutions.

Discussion. The bactericidal assay for antibodies against Gram-negative bacteria is most useful in tests on normal sera where the individual antibody specificities are present in very low concentration. This procedure is founded on the principle that, in the presence of complement in excess, the extent of bactericidal activity provided by a test serum is dependent on its content of specific antibody. However, the manner in which the test is set up and performed can be varied considerably. Perhaps the most important consideration is selection of an appropriate complement source, *i.e.*, one lacking the antibody to be measured. The choice lies between the use of fresh normal serum of adult mammals, or exploiting those special situations in which mammalian serum normally is lacking in these antibodies. In the former it is necessary to remove the antibodies invariably present, whereas in the latter situation (serum from newborn and germfree animals) the need for such manipulations frequently is obviated.

A number of reports(6,8,9) suggest that precolostral calf serum is deficient in these natural antibodies. Accordingly, the possibility was considered that such sera could meet the requirements for an antibody-free complement source. In the present work a series of 30 individual calf serums were tested for bactericidal activity against bacterial strains representing 6 Gram-negative genera. From this survey the following picture emerged. The antibodies consistently absent were those against *E. coli, A. aerogenes* and *Pseud. aerugenosa.* A modest proportion of the sera was free of antibody against *S. typhosa* and *Shig. dysenteriae,* but *all* the sera were active against *Kleb. pneumoniae.* These findings had not been anticipated on the basis of previous work. Nonetheless, these sera have proved to be uniformly satisfactory for assay of certain antibodies such as those against *E. coli, P. aerugenosa* and *A. aerogenes.* Providing they are first screened to determine whether antibodies are present, they can also be used to test for still other specificities. We have explored the possibilities offered by precolostral calf serum in providing complement without certain antibodies because of the advantage of availability in rather large amounts and low cost. Other large mammals such as sheep and swine also possess a placental barrier impermeable to antibodies. The number of individual specificities is significantly reduced in these 2 species; however, some specificities were nonetheless discernible in the newborn, colostrum-free state. The distribution of antibodies appears to be different from that found in the calf. These findings will be reported elsewhere. It would, of course, be a great convenience if the precolostral serum of any single species of large mammal were to lack all of the various Gram-negative antibody specificities, while providing functional complement. On the basis of our experience this possibility seems rather unlikely. However, by appropriate selection of serum from the newborn calf, piglet or lamb, it is entirely feasible to obtain complement suitable for the assay of antibody against most of the *Enterobacteriaceae.*

In an assay for antibody based on the use of a very small bacterial inoculum rather than a large one it might be expected that less antibody would be required to produce 50% killing. This would be the case if the amount of antibody required for the reaction was proportional to the size of the inoculum. Reports by Sterzl *et al.*(10,11) on the bactericidal reaction and its use in detection and measurement of antibody indicate that these workers interpreted their findings along these lines. In the present work, however, the data provided by experiments with inocula which differed in amount by 10,000-fold showed that in these opposing situations the *quantity* of antibody required for 50% bactericidal effect was, for all practical purposes, the same. These findings agree with the report by Andrews and Elford(12) that the same proportion of viruses were inactivated by a given concentration of serum, whether the inoculum

was as small as 10^2 or as large as 10^8 particles per ml. Similar results were obtained by Muschel and Toussaint in their study of the effect of normal human serum on coli T_2 phage(13). Thus the "percentage law" which these workers(12) established for mammalian and bacterial viruses appears also to apply to bacteria.

What is significant is that the *number* of bacteria killed is dependent upon size of the inoculum, whereas the *percentage* killed is a function of concentration of antibody. Consequently, a reduction in size of the inoculum, even by a factor of 10,000-fold, does not appreciably increase the sensitivity of the test. It does, however, eliminate the labor associated with serial dilution and plating, greatly facilitating execution of the assay.

Statistical analysis of 15 replicate titrations on pooled normal rabbit serum suggests that variation from one experiment to another is appreciably greater than within individual experiments. This is supported by the finding that no point exists on the scale of concentrations utilized that is covered by as few as half of the fifteen 95% confidence intervals. Had the variation within experiments been larger, the corresponding confidence intervals would have increased in length and would have tended to overlap.

Since the sight estimates are in accord with the calculated estimates of the SD_{50}'s the former method of obtaining this value has been employed routinely. The data leave no doubt that this test for the various Gram-negative specificities is highly sensitive. In practice SD_{50} values showing differences greater than 2-fold have been considered significant as based on the range of values obtained with replicate titrations of a single serum.

There is an increasing awareness that the antibody specificities in normal serum directed against Gram-negative species probably influence host reactivity to Gram-negative bacteria and their endotoxic polysaccharides. The paucity of existing information in this regard has been due largely to lack of appropriate methodology for detection and measurement of these antibodies as found in normal serum(4). The basic requirements for such a method are precision, high sensitivity, and ease of performance. Earlier studies in this laboratory(3) had led to the development of an assay procedure, based on the bactericidal reaction, which met reasonably well the needs for precision and sensitivity, but was rather cumbersome and tedious in execution. The changes here proposed should serve to simplify greatly those aspects of the procedure which have tended to limit the extended application of the assay. The simplified method lends itself to routine laboratory use and may facilitate investigation of the problems associated with the origin and properties of natural antibodies.

Summary. A bactericidal assay for antibodies against smooth strains of enterobacteria has been described which differs from earlier versions in several aspects. Precolostral calf serum was utilized as a "natural" source of complement lacking certain antibodies. The inoculum size was reduced to 10^2 cells. Expedient and more direct means were devised for determining the results of the interaction of complement test serum (antibodies) and bacteria. These changes made possible a great economy of effort without sacrifice of sensitivity and precision.

The competent technical assistance of Mr. Carlcs Fernandez, Mr. Walter F. Kearney, and Mr. Frank Koczot is gratefully acknowledged. Dr. David W. Alling, NIAID, NIH, kindly performed the statistical evaluation of data.

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Conversion of Diopterin and Teropterin into Folic and Folinic Acid Activity by the Rat Liver.* (28514)

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The synthetic folic acid conjugates pteroyl-alpha-glutamylglutamic acid (Diopterin) and pteroyl-gamma-glutamyl-gamma-glutamylglutamic acid (Teropterin), respectively, have been effective in treating the megaloblastic anemia of sprue(1) and pernicious anemia (2). Such hemopoietic effects probably result from conversion of these conjugates into folic acids active for man(2). Although Diopterin (1) and Teropterin(3) are partially excreted in the urine as free folic acid, neither the sites of conversion nor the exact nature of the resulting products are known.

The present studies were therefore carried out to determine the liver's ability to transform Diopterin and Teropterin into products having folic and folinic acid activity and to see if differences in structure of the two molecules cause one to be more effectively converted than the other. For comparison purposes, the reduction of pteroylglutamic acid (PGA) to folinic acid was studied according to the same protocol.

Materials and methods. Three groups of Sprague-Dawley rats, each containing 3 animals, were maintained on Purina Laboratory Chow. The livers of all rats were removed and perfused in an apparatus adapted from the one described by Miller(4). One liver from each group was perfused from a reservoir of 200 ml heparinized blood containing a starting concentration of either Diopterin (6.4 μg per 100 ml) Teropterin (7.3 μg per 100 ml), or PGA (5.0 μg per 100 ml). Blood flow was maintained at a constant rate of 60 drops per minute. The minimum bile flow was 0.2 ml per hour. Blood samples were collected, 15, 45, 75, and 105 minutes from the beginning of perfusion from a filter proximal to the liver inflow cannula. At the end of each perfusion the liver was placed in formalin; and histologic sections, stained with hematoxylin and eosin, were prepared. Blood was diluted 1:1 with 0.05 M sodium phosphate buffer. The blood-buffer solution was autoclaved for 10 minutes at 118°C and then centrifuged for 5 minutes at 3000 rpm to remove coagulum. Clear supernatant was removed and used for folic acid activity assay with *Lactobacillus casei* and *Streptococcus faecalis* and for folinic acid activity assay with *Pediococcus cerevisiae*(5). Conversion of Diopterin to folic acid activity was expressed as per cent increase of *L. casei* activity in perfusing blood. For Teropterin this same calculation was made using *S. faecalis* activity, since this conjugate is 60 to 80% as active when assayed with *L. casei* but only 2 to 6% as active when assayed with *S. faecalis* as is PGA itself(6). Conversion of PGA, Diopterin, and Teropterin to folinic acid activity was quantified as per cent increase of *P. cerevisiae* activity in perfusing blood. Increase of activity in each case was expressed as percentage of starting concentration. Diopterin and Teropterin were assayed *in vitro* in the concentrations employed in these experiments using both *L. casei* and *S. faecalis*. Diopterin supported growth of neither organism. Teropterin permitted growth of *L. casei*, as expected, but did not permit growth of

* This study was supported in part by grants from Nat. Inst. Health.