

THE ESTIMATION OF THE BACTERICIDAL POWER OF THE BLOOD

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THE killing power of the blood is usually estimated from the result of cultivating mixtures of whole or defibrinated blood with graded inocula of bacterial culture. A series of culture dilutions made with a constant dilution factor provides what is in effect a logarithmic scale upon which the bactericidal power may be measured in terms of the number of dilutions sterilized (e.g. Mackie & Finkelstein, 1931). In many tests, however, the bactericidal power of the blood is of a lower order; the blood sterilizes only the highest dilution of culture that contains viable organisms, or the viable organisms in a given dilution undergo only a reduction in numbers. It would be unjustifiable, from the result of two such tests, to infer anything about differences in the bactericidal power of two bloods without some knowledge of the sampling errors inherent in the technique employed.

The absence of growth from a small inoculum in blood cannot be depended upon as an indication of killing, unless very large numbers of fluid cultures are made from each blood-inoculum mixture (Fisher, 1930, section 17).¹ Within the limits of a practicable technique, accuracy is approached only when methods involving colony counts are used. The investigations described below were directed particularly towards finding reliable quantitative expressions for the killing power of human blood for *Staphylococcus aureus*, measured by viable counting methods, but many of the principles involved are applicable to bactericidal tests in general.

THE ESTIMATION OF THE NUMBER OF BACTERIA INOCULATED

Two accurate viable counting methods are available: the plate count and the roll-tube count. Both, depending on the incorporation of the culture in molten agar media, involve a number of laborious manipulations. We have used a similar count of the colonies developing from measured drops of culture dilutions seeded on the *surface* of well-dried agar plates. A few parallel tests have shown that this "surface-viable" count does not differ significantly from counts of colonies developing in the depth of roll-tubed agar. We have not

¹ All subsequent references to Fisher indicate various sections of the 1930 publication.

pursued this point, for "viability" is a function of the cultural conditions, and provided that these are optimal and standard for the technique employed, the actual level of viability, within reasonable limits, is immaterial. It remains to be tested whether a series of surface-viable counts gives results as consistent as those of the established plate and roll-tube counts.

Method. Dropping pipettes, giving 50 drops of broth to the c.c., were employed. Ten-fold dilutions of culture are made, 0.1 c.c. being added to 0.9 c.c. of diluent broth, and a fresh pipette used for each dilution. (For details of the pipette technique and its reliability see Wilson, 1922, 1935.) Two per cent. agar plates, after the routine 24 hr. drying at 37° C. with the lids closed, are dried for a further 2 hr. with the lids raised. Except for counts of delicate bacteria, this period may be extended to 6 hr. without materially altering the count result. The plates should be dry enough to absorb a 0.02 c.c. drop in 15–20 min., and for this a standard 2 hr. drying is usually sufficient. On such plates, 0.02 c.c. of broth, dropped from a height of 2.5 cm., spreads over an area 1.5–2.0 cm. in diameter. Six to eight dilutions of culture are made, and at the same time each of six plates receives one drop of each of the dilutions in numbered sectors; after the absorption of the drops the plates are incubated in the usual way. The drop areas from the higher concentrations of culture yield circular patches of confluent growth. Counts are made in drop areas containing the largest number of colonies without signs of confluence or of gross diminution in colony size due to overcrowding. The number of colonies is estimated from the mean of the six counts. With large colonies of organisms like *Bacterium coli* (Kenny *et al.* 1937), the maximum number obtainable without confluence in the drop area may be as low as 20, but with small colonies, like those of *Staphylococcus aureus* after 18 hr. growth, counts up to 100 per drop area may be made.

Tests of a suitable medium

Ideally, the frequencies of constant-volume samples of a thin bacterial suspension containing 0, 1, 2, 3, etc., living bacteria should conform to a Poisson series $e^{-M}(1 + M + M^2/2! + M^3/3! + \dots \text{etc.})$, where M is the average bacterial content per sample. The viable counts from such samples will also conform to the Poisson series if (1) each plate offers the same facilities for the development of each organism; (2) the development of any organism is independent of other organisms present, and if development results only in one visible colony; (3) each plate has an equal chance of receiving any organism, and if the organisms are distributed independently. These conditions were enumerated by Fisher *et al.* (1922) for counts of mixed bacterial populations. They are easier to fulfil for surface-viable counts of pure cultures. (2) is satisfied if there is no overcrowding in the drop areas, and if, as with the strains of *Staph. aureus* used, the organisms are dispersed singly in the culture. (3) is realized in the technique of diluting and distributing the drops. There remains condition (1), and it follows that, other conditions being satisfied, an imperfection of nutrient qualities of the medium would be reflected, not only in a reduction of the number of bacteria developing into colonies, but in a lessened conformity of the six count values with the ideal Poisson distribution.

The mean count, M , and the standard error, s.e., of a count may be estimated from six parallel platings, x_1, x_2, \dots, x_6 , by the expressions $m = S(x)/N$, and $\sigma = \sqrt{[S(x - m)^2 / (N - 1)]}$ respectively, where N is the number of plates.

The s.e. of an ideal Poisson distribution is equal to the square root of the mean, and some idea of the conformity of a particular count to the Poisson series can be gained by comparing the calculated s.e. with the expected s.e., \sqrt{m} . But this comparison does not indicate the degree of conformity, and the goodness of the fit of the six-plate count to a Poisson series may be more accurately measured by the χ^2 test. χ^2 is calculated, according to the formula for small samples of a Poisson series (Fisher, sect. 16), from the relation $\chi^2 = S(x-m)^2/m$. With only six available values of x , the resulting χ^2 is not a reliable indication of conformity, but the values of χ^2 from a number of such counts should fall within assignable limits if the counts themselves are derived from Poisson variates. Parallel counts were made on nutrient agar plates (N.A.P.) prepared according to Hedley Wright's (1933) method, and on the same medium containing 2% Fildes' extract. In all, sixteen batches of ox-heart extract were

Table I. *The distribution of χ^2 from surface-viable counts of Staph. aureus on nutrient and Fildes' agar plates*

χ^2	Expected	F.A.P.					
		F.A.P.	N.A.P.	Expected	Total	$m < 20$	$m > 35$
0.00- 0.55	0.16	—	—	0.94	1	—	1
0.56- 0.75	0.16	—	—	0.94	2	—	2
0.76- 1.15	0.58	—	—	2.82	2	1	1
1.16- 1.61	0.80	2	—	4.7	6	2	4
1.62- 2.34	1.6	1	2	9.4	12	6	6
2.35- 3.00	1.6	—	1	9.4	8	5	3
3.01- 4.35	3.2	6	3	18.8	20	10	10
4.36- 6.06	3.2	3	2	18.8	22	13	9
6.07- 7.29	1.6	1	2	9.4	5	4	1
7.30- 9.24	1.6	1	2	9.4	7	4	3
9.25-11.01	0.80	1	2	4.7	6	5	1
11.02-13.39	0.58	1	—	2.82	2	2	—
13.40-15.09	0.16	—	1	0.94	1	—	1
15.10-	0.16	—	1	0.94	—	—	—
	16.00	16	16	94.00	94	52	42

tested. The conformity of the sixteen counts with the Poisson distribution is illustrated in Table I, cols. 2-4. The distribution of χ^2 from the N.A.P. and Fildes' counts is compared with the theoretically expected distribution (Fisher, sect. 24). The number of counts is too small to test the fit very well, but the predominance of unduly high values of χ^2 from the N.A.P. results is suggestive. In a number of parallel counts that are not included in this analysis, one or more of the six N.A.P. yielded a widely discrepant count, 10-, 100- or a 1000-fold less than the mean of the remaining plates; the plates were all made from the same batch of medium in each case, and no obvious cause, such as the thickness or the pH of the agar, could be found for the discrepancy. The Fildes' plate results, on the other hand, were remarkably constant throughout.

A second possible criterion of the value of a medium is the size attained by discrete colonies after standard incubation. The most luxuriant growth, however, is not necessarily obtained on media with the greatest capacity to initiate growth. For example, a series of tests, made in another connexion, comparing counts of *Brucella melitensis* on nutrient and on liver agar, showed that some batches of liver medium failed to support the growth of an

inoculum that yielded 100 colonies on nutrient agar; yet the growth on liver from 500 nutrient-agar-viable organisms was four times as luxuriant as that of the same inoculum on the nutrient agar. Nevertheless, well-developed colonies are useful indications of the goodness of a medium, if judged in conjunction with the count values.

A third criterion is available in the variation in colony size on different media. In general, the worse the medium, the greater the variation. The wide variation induced by the addition to a medium of a partially effective concentration of a bacteriostatic substance is obvious without measurement (e.g. the growth of *Brucella abortus* on weak thionin media). In less obvious instances, differences in variability are revealed by measurement. For example, we have found that the magnitudes of the coefficient of variation of *Meningococcus* colony sizes on serum and on haemoglobin agar, in air and in air + 10% CO₂, increased in the same order as the mean colony size and mean count decreased; i.e. Hb agar in CO₂ > serum agar in CO₂ > the Hb agar in air > serum agar in air. Moreover, in these tests and in a series of tests of *Haemophilus parainfluenzae* on blood and Fildes' medium, the diameters of the largest colonies on the medium with the greater scatter seldom exceeded the mean diameter of those on the small-scatter medium; i.e. the scatter was in the direction of small colonies, indicating a defect of the one medium that resulted in a growth-lag of the less vigorous organisms.

The colony sizes on the six-plate counts were measured under low-power magnification in drop areas containing two to five colonies, and the mean, \bar{x} and s.e. were calculated from fifteen to thirty measurements. The coefficient of variation, c.v., expressed as a percentage, was chosen as the index of scatter: $c.v. = (s.e. \times 100) / \bar{x}$.

Table II. Mean colony counts and mean colony sizes of *Staph. aureus* on 16 batches of nutrient and Fildes' agar

Count			Colony size (mm.)				
N.A.P.	F.A.P.		N.A.P.		F.A.P.		
m_1	m_2	m_2/m_1	\bar{x}_1	C.V.	\bar{x}_2	C.V.	\bar{x}_2/\bar{x}_1
2.6	3.6	1.49	—	—	—	—	—
4.2	7.3	1.74	—	—	—	—	—
4.7	6.3	1.34	—	—	—	—	—
5.7	5.2	0.90	—	—	—	—	—
7.7	8.3	1.08	1.04	20.0	1.06	9.3	1.02
11.6	15.0	1.35	0.69	10.1	0.78	10.7	1.13
13.3	17.0	1.28	0.56	28.2	0.95	10.3	1.70
13.3	12.8	0.96	1.43	10.0	1.45	6.2	1.01
14.1	16.8	1.19	1.34	13.3	1.45	6.2	1.08
14.3	17.1	1.20	1.69	8.1	1.69	11.1	0.00
25.7	79.2	3.16	1.67	6.0	1.72	5.7	1.17
34.5	37.6	1.09	1.08	9.3	1.15	4.0	1.06
39.0	46.5	1.18	1.63	6.7	1.71	3.9	1.05
47.2	73.0	1.55	2.01	5.0	1.90	5.9	0.95
51.5	60.0	1.18	0.94	12.3	1.04	9.4	1.13
53.3	60.2	1.14	—	—	—	—	—

Of the sixteen batches of media (see Table II), fourteen gave a slightly higher Fildes' count. In the eleven tests of colony size the Fildes' colonies were larger in nine, the same size in one, and less in one. The differences between the corresponding pairs of means, either of counts or colony size, were not individually significant. (The significance of the differences was assessed by the *t* test, where $t = \text{difference} / \text{standard error of difference}$.) But by calculating the F.A.P. : N.A.P. ratios, both of counts and sizes and treating these as Fisher (sect. 24), the increase in count and mean diameter with the addition of Fildes' extract was such that it would occur by chance once in twenty and

ten trials respectively, on the assumption that the media did not differ in their effect on *Staph. aureus*. The average coefficient of variation of colony size was 12.9% on N.A.P. and only 8.2% on Fildes; in three of the eleven tests the c.v. was greater on the Fildes, though only by 0.6, 0.9 and 3.0%. By all the criteria, Fildes' agar was indicated as the medium for *Staph. aureus*. Fildes' extract, which can be prepared in bulk and kept for some time, apparently acts as a standardizing substance, bringing the more variable heart extract up to a definite level of excellence.

The choice was justified by the analysis of subsequent Fildes' counts. Table I, col. 6, shows the distribution of χ^2 calculated from ninety-four six-plate Fildes' counts of *Staph. aureus*. The fit with the expected distribution in col. 5 is good, for the total χ^2 from all counts differs from its expected value, $\sqrt{(2\chi^2)} - \sqrt{(2n-1)} = 0$, by -0.4733 (there are $94(N-1) = 470$ degrees of freedom), a deviation to be expected 32 times in 100 such trials. The means of the counts fell into two groups, one containing low values between 2 and 20, the other high values between 35 and 125. A separation of the values of χ^2 from these groups yielded the distributions in cols. 7 and 8. There is a greater spread of values from the higher means, an effect ascribable to overcrowding. It is only slight, because the $m > 35$ distribution shows no marked deviation from the expected distribution. Nevertheless, in practice, drop-area counts of 80 or over were avoided in favour of the counts of 8 or more which arose from the drop areas seeded with the next lower tenfold dilution.

The detailed analysis of the data presented above not only gives several independent indications of the comparative value of a medium, but it provides a sound basis for simplification of the calculations involved in dealing with sampling errors. For if it can be established that the technique yields counts conforming with samples from a Poisson series, the calculation of the s.e. from the individual values of a count may be dispensed with, and assumed to be equal to the square root of the mean count. We shall deal with this point in connexion with the s.e. of the bactericidal power.

THE MEASUREMENT OF BACTERICIDAL POWER

Technique of the bactericidal tests

Defibrinated blood was distributed in 0.4 c.c. lots in six soft-glass tubes 70×5 mm. internal diameter. The blood was tested within half an hour of its withdrawal from a vein. Each tube received five 0.02 c.c. drops of each of six culture dilutions, and at the same time samples were taken from the dilution tubes for the surface-viable count, thus ensuring a count of the inoculum at the time of mixing with the blood. The resulting 0.5 c.c. fluid column occupied about 20 mm., leaving ample room for the motions of the fluid during mixing. The tubes were sealed with glass plugs cemented in with hard wax (R. Hare, personal communication), mounted in a Todd's (1927 *a*) mixing machine, and incubated at 37° C. After incubation, the contents of the tubes were sampled with standardized dropping pipettes, and six 0.02 c.c. drops of each mixture let fall on to numbered sectors of six well-dried Fildes' agar plates, as in the surface-viable count. Absorption of the blood fluid took place, leaving on the agar surface an opaque red gel in the form of a thin plaque about 15 mm. in diameter (referred to

below as the "plaque"). The development of staphylococcal colonies in the depths of this gel was slow, and the final size attained by the colony small, but at its edge or on its surface the growth was as rapid as that on a plain agar medium.

Counts of the same plaque made after 18 and 42 hr. incubation were not significantly different individually, though 42 hr. counts were always higher. In fourteen comparisons of six-plate means, the ratio of 18 to 42 hr. counts ranged from 1:1.02 to 1:1.5, with a mean of 1:1.15, and the difference (1.0-1.15 = -0.15) was 2.75 its own standard error. From the *t* test this difference would arise by chance only once in 70 times, so that the 42 hr. counts, as a whole, were significantly higher. 42 hr. counts were used in all the following determinations.

Table III. *The distribution of χ^2 from plaque counts of Staphylococcus blood mixtures, on Fildes' agar*

χ^2	Total		$m < 5.0$		$m > 5.1$	
	Expected	Observed	Expected	Observed	Expected	Observed
0.00- 0.55	1.79	2	0.79	1	1	1
0.56- 0.75	1.79	4	0.79	2	1	2
0.76- 1.15	5.37	20	2.37	8	3	12
1.16- 1.61	8.95	11	3.95	3	5	8
1.62- 2.34	17.9	26	7.9	13	10	13
2.35- 3.00	17.9	21	7.9	5	10	16
3.01- 4.35	35.8	40	15.8	21	20	19
4.36- 6.06	35.8	22	15.8	9	20	13
6.07- 7.29	17.9	17	7.9	11	10	6
7.30- 9.24	17.9	11	7.9	5	10	6
9.25-11.01	8.95	5	3.95	1	5	4
11.02-13.39	5.37	0	2.37	0	3	0
13.40-15.09	1.79	0	0.79	0	1	0
15.10-	1.79	0	0.79	0	1	0
	179.00	179	79.00	79	100	100

The plaque counts from 179 six-plate determinations were tested for their goodness of fit to a Poisson series. Table III, cols. 2 and 3, shows the distribution of χ^2 . There is a marked excess of low values over the high values. The total χ^2 from an unselected series of 144 of these counts was 534.10, and $\sqrt{(2\chi^2)} - \sqrt{(2n-1)}$ is -5.25 (there are 144 ($N-1$) = 720 degrees of freedom), indicating a significant deviation from the Poisson series. A general anti-bacterial property of the plaque, such as the persistence of the bactericidal power of the blood after its removal from the tube, would suppress organisms whose vigour lies below a certain level, and the remaining organisms destined to produce visible colonies would still give Poisson counts. The cause of the deviation must therefore be sought for in a variability of growth-promoting properties in different parts of the plaque, such that there is a selective action on certain samples or parts of samples. If, for example, some of the organisms in high-value samples fail to develop, whereas all the organisms develop in low-value samples from the same mixture, the result would be a reduction of the standard error of the count from all the samples and a drop in the value of χ^2 obtained from it. The selective action is not associated with overcrowding of

colonies in the plaque, at any rate in the range studied, for the χ^2 values are not materially lessened with increasing numbers of colonies in the plaques. The 179 χ^2 values in Table III have been divided into two distributions (cols. 4-7), i.e. those from mean plaque counts up to 5.0, and those from counts 5.1 to 75, and both show similar degrees of asymmetry. The same result was indicated by the series of 144, which, divided into 72 high- and 72 low-value counts, gave χ^2 totals of 261.58 and 272.52 respectively, differing by the insignificant amount of 10.94 for 360 degrees of freedom.

There are two practical consequences of this result, whatever its cause may be. In the first place, the low χ^2 values indicate that the mean number of organisms surviving the exposure to blood may in fact be greater than the number of colonies that develop in the plaques. In the second place, the square root of the mean will not be as reliable an estimate of the standard error of the plaque counts as it was of the drop-area counts of culture dilution.

The estimation of the bactericidal power

Bactericidal power is most conveniently indicated by p , the proportion of n bacteria that survive mixture with the blood tested. $p=r/n$, where r is the number of bacteria surviving, and the bactericidal power is inversely proportional to the survival rate p .

The estimates of r and n are derived from means of surface-viable and plaque counts, both of which are subject to sampling errors. The standard error of p is

$$\sigma_p = \sqrt{(pq/nN + p^2\sigma_n^2/n^2)} \quad \dots(i)$$

(see Dr Irwin's note), where $q=(1-p)$, σ_n is the standard error of n and N the number of plates used in the determination of r and n .

The χ^2 data justify the use of \sqrt{m} as a reliable estimate of the standard error of the surface-viable counts, and of $\sqrt{m/N}$ (where N =the number of plates) as an estimate of the s.e. of the mean count. If $m=n$, $\sigma_n^2=n/6$ and $\sigma_p = \sqrt{(pq/6n + p^2/6n)}$.

The standard error, σ_r , of the mean of the plaque-counts does not appear in equation (i), and as far as the actual calculation of σ_p is concerned, need not be known. But p is derived from r , and the standard error of r will in part determine the reliability of p as an estimate of the number of bacteria surviving. The χ^2 data showed that

- (1) the standard error of the plaque counts was usually less than \sqrt{r} , the standard error to be expected if r was derived from a Poisson series, and that
- (2) the mean of the plaque count was probably an under-estimate of r , the number of bacteria surviving.

As far as (1) is concerned, by adopting $\sqrt{r/N}$ ($=\sqrt{r/6}$) as the value for r , we should, in the majority of cases, be using a larger standard error than that derived from the actual plaque counts, and consequently be increasing the value of a standard error, such as that of p , derived indirectly or directly from that quantity. As a result of (2), p will be over-estimated. The over-estimation will, however, be consistent, for the skewness of the χ^2 distribution was independent of the magnitude of the mean plaque-counts occurring in the tests. Our object is a comparison of bactericidal powers, and a *consistent* degree of under-estimation of r will

not affect the comparison. We have therefore made the assumption that the mean plaque count is a reliable estimate of r , and that r has a standard error of $\sqrt{r/6}$, though this second value is not used explicitly in our calculations.

Table IV. *Relations of formulae for the calculation of p and σ_p from the mean counts m in different dilutions of test culture, and r , the mean plaque count. "U" indicates an uncountable number of colonies in the drop areas*

Culture dilution used (say)	10^{-4}	10^{-5}	10^{-6}
Mean of N (=6) colony counts in 0.02 c.c. culture-dilution	"U"	"U"	m
Estimated number of organisms in 0.1 c.c. of dilution	$500m$	$50m$	$5m$
Inoculum (n) in 0.02 c.c. blood-organism mixture ($n = km$)	$20m$	$2m$	$m/5$
S.E. of inoculum (σ_n)	$20\sqrt{m/N}$	$2\sqrt{m/N}$	$\sqrt{m/25N}$
$\therefore \sigma_n =$	$\sqrt{20n/6}$	$\sqrt{2n/6}$	$\sqrt{n/30}$
Survival rate (p)	$r/20m$	$r/2m$	$5r/m$
S.E. survival rate (σ_p)	$\sqrt{[(p + 19p^2)/6n]}$	$\sqrt{[(p + p^2)/6n]}$	$\sqrt{[(p - 0.8p^2)/6n]}$

In the experimental conditions, the mean of the surface-viable count, m , is not in fact the value of n , the blood inoculum in the test proper. For convenience of description, let the countable number of colonies, m , arise from 0.02 c.c. of a 10^{-6} dilution (see Table IV); 0.1 c.c. is added to 0.4 c.c. blood, and each blood-drop of 0.02 c.c. contains $m/5$ organisms. Since bloods of low bactericidal power kill at least 50% of the cocci, very few organisms develop from such a mixture, and the mean plaque count is unreliable. The 10^{-5} dilution of the staphylococcal culture is uncountable (Table IV, "U") and the drop content is therefore estimated from the 10^{-6} count as $10m$; blood drops from mixtures with this dilution contain $2m$ cocci. Similarly, blood drops made with the 10^{-4} dilution contain $20m$ cocci. Thus n may have the values $m/5$, $2m$, $20m$, etc., with standard errors $\frac{1}{5}\sqrt{m/6}$, $2\sqrt{m/6}$, $20\sqrt{m/6}$, etc. The standard error of n , σ_n , will be $\sqrt{n/30}$, $\sqrt{2n/6}$, $\sqrt{20n/6}$, etc., according to the dilution of culture used for the estimation of m . Substituting these values of n in equation (i) we have

$$\text{for } n = 0.2m, \quad \sigma_p = \sqrt{[(p - 0.8p^2)/nN]}, \quad \dots(\text{ii})$$

$$n = 2m, \quad \sigma_p = \sqrt{[(p + p^2)/nN]}, \quad \dots(\text{iii})$$

$$n = 20m, \quad \sigma_p = \sqrt{[(p + 19p^2)/nN]}. \quad \dots(\text{iv})$$

The relations of these various values are shown in Table IV.

Given p and σ_p , the significance of a difference, $d = (p_1 - p_2)$, between any two values p_1 and p_2 of the survival rate in two bloods, can be estimated in the usual way (Fisher, sect. 24.1). The standard error of the difference, σ_d , is $\sqrt{(\sigma_{p_1}^2 + \sigma_{p_2}^2)}$, and if d/σ_d is less than 3, the difference cannot be assumed to have arisen from any significant difference in the bactericidal powers of the two bloods.

As examples of the calculations, the figures in Table VIII A, tests 28 and 33, were obtained as follows. In test 28, m was 40.7 in the 10^{-5} culture dilution. The plaque count, r , from the mixture with the 10^{-3} culture-dilution, was 18.8. n is therefore $20m = 814$, and $p = 18.8/814$

$=0.0231$; σ_p^2 is $[0.0231 + 19(0.0231)^2]/6 \times 814 = 0.000006805$ (equation (iv)). In test 33, $m = 69.3$ in the 10^{-5} dilution; $r = 8.2$ in the mixture with the 10^{-4} culture-dilution; therefore $n = 2m = 138.6$, and $p = 0.0591$; $\sigma_p^2 = [0.0591 + (0.0591)^2]/6 \times 138.6 = 0.00007526$ (equation (iii)). The difference between the two values of p is 0.0360 , and $\sigma_d = \sqrt{(0.000006805 + 0.00007526)} = \pm 0.00906$. The difference, in terms of its own standard error is $d/\sigma_d = 0.0360/0.00906 = 3.97$, and is therefore significant in the sense defined above.

STANDARDIZATION OF THE TEST

Rotation period. Five parallel sets of bacterial test-tubes of the same specimen of blood were rotated with the same culture dilutions at 37°C . for 1, 2, 4, 6 and 24 hr. respectively. Table V summarizes the result of three experiments with normal bloods of different bactericidal powers. Maximum killing takes place at 2 hr.; staphylococci multiply slowly up to 6 hr. and are growing freely at 24 hr.

Table V. *Effect of time of rotation on p; three normal human bloods.*
Strain P 900

Time hr.	Experiment		
	1	2	3
1	0.1610	0.546	0.1790
2	0.0675	0.448	0.0413
4	0.0733	0.675	0.0595
6	0.0745	(1.850)	0.0762
24	Overgrown	Overgrown	Overgrown

Strains used. Two strains of *Staph. aureus* were used, P 802 and P 900. They were recently isolated from acute abscesses, fermented mannitol, liquefied gelatin rapidly and were coagulase and Voges-Proskauer positive. Subcultures of P 802, used in the technical tests, were grown each time from 0.75% nutrient agar stab cultures that had been kept at room temperature and subcultured every 6 weeks. Strain P 900 was standardized as follows for the bactericidal tests of human blood. The centrifuged deposit of a young broth culture was distributed into a large number of tubes containing sterile filter paper. The papers were dried *in vacuo* over H_2SO_4 and the tubes sealed with wax and stored in the dark at room temperature. When required, the dry culture was incubated for 18 hr. with 5 c.c. broth, and a subculture from this used for the tests.

Age of culture. Parallel tests were made to determine the susceptibility to blood of 3 and 18 hr. cultures, both washed and unwashed. Washing consisted of centrifuging the culture and resuspending the packed and drained sediment in fresh warm broth. Tables VI A and B summarize the results of these tests. Three-hour cultures are killed less easily than 18 hr. cultures. The work of Lyons (1937) suggests that in 18 hr. cultures of *Staph. aureus*, the cocci are more degraded than in 3 hr. cultures. The use of 3 hr. cultures is thus indicated in routine bactericidal tests. But since the differences between each of the two values of p in Exps. 4, 5 and 6 is not significant, the more convenient 18 hr. culture was used in the majority of the tests. The effect of washing either the 3 or the 18 hr. cultures is variable (Exps. 7-12, Table VI B).

Table VI A. *Effect of the age of the culture of p; normal human blood.*
Strain P 802

Exp.	3 hr. culture		18 hr. culture		σ_d	d/σ_d
	p_1	p_2	p_1	p_2		
4	0.212	0.156	+0.046	0.0247	1.86	
5	0.238	0.197	+0.041	0.0288	1.42	
6	0.180	0.131	+0.049	0.0226	2.17	

Table VI B. *Effect of washing the culture on the value of p; normal human blood. Strain P 802*

Exp.	Age culture hr.	Unwashed p_1	Washed p_2	d	σ_d	d/σ_d
7	18	0.139	0.163	-0.024	0.0177	1.35
8	18	0.144	0.186	-0.042	0.0208	2.02
9	18	0.422	0.334	+0.088	0.0310	2.83
10	3	0.182	0.130	+0.052	0.0191	2.72
11	3	0.104	0.110	-0.006	0.0208	0.29
12	3	0.080	0.056	+0.024	0.0143	1.68

In all the following experiments, the blood was mixed with unwashed 18 hr. cultures, rotated at 37° C. for 2 hr., and the plaque colonies counted after 42 hr. incubation.

It might be objected that the plaque count measures not only the bactericidal effect of the blood up to the moment of sampling, but also that of the concentrated semisolid blood of the surface of the agar plate. Todd (1927*b*), using streptococci, virtually stopped the bactericidal effect at the moment of sampling by diluting the blood-bacterium mixture in molten agar. For an estimation of the *rate* of killing such a stoppage is essential, either by dilution or by the addition of a neutralizing substance. Experiments with sodium poly-anetholsulphonate (Liquoid) show that it can be used in this way to neutralize a high proportion of the total bactericidal powers of rabbits' blood; and culture experiments in human blood (von Hæbler & Miles, 1938) indicate similar degrees of neutralization. In the recorded experiments the samples were taken at the time of maximum bactericidal effect, after which further incubation in the rotation tubes resulted in multiplication of the cocci. At this stage little bactericidal power remains, for in a small series of tests the plaque count was not significantly altered by the addition of 0.05 % of Liquoid to the blood-bacterium mixture immediately before sampling for the plaque count.

FALLACIES IN THE INTERPRETATION OF p

The number of colonies developing in the blood plaques may be decreased as a result of aggregation of the cocci by at least two mechanisms, phagocytosis and agglutination.

Aggregation by phagocytosis. Two or more organisms, each destined to survive as a colony producer whether ingested by a leucocyte or not, may be gathered into one leucocyte and give rise to one colony only.

Some idea of the magnitude of this error may be gained from the following considerations. We may assume highly favourable conditions for phagocytosis, i.e. that the blood contains as many as 20,000 equally phagocytic w.B.C. per c.mm., that contact with a coccus results in its ingestion by the w.B.C., and that all the cocci are so ingested. The number of such w.B.C. in 0.02 c.c. will be 400,000. An inoculum of 400 cocci in this volume represents an average bactericidal mixture. Initially there are 1000 w.B.C. to each coccus, and since phagocytosis cannot take place in the free fluid except by chance approximation of w.B.C. and coccus, the probabilities, P , of any given w.B.C. ingesting

0, 1 or 2 bacteria will be the first three terms of a Poisson series, in which $M=1/1000$, the ratio of bacteria to w.B.C. These are:

No. ingested	0	1	2
<i>P</i>	0.99900	0.0009990	0.00000119950

i.e. the odds against one leucocyte ingesting two organisms are over 2 million to 1. But if an inoculum large enough to equal the number of w.B.C. is used, the probabilities are as follows:

No. ingested	0	1	2	3
<i>P</i>	0.3679	0.3679	0.1839	0.0613

i.e. 18% of the cells may contain two, and 6% may contain three organisms.

Two rotation experiments, mixing *Br. melitensis* with defibrinated rabbit blood and a highly opsonic anti-*melitensis* serum, illustrated the effect of non-lethal phagocytosis on large and small inocula. There was no reduction in count of the small inocula of 5–40 per 0.02 c.c.; *p* in fact was 1.0. But the blood plaques from mixtures incubated with 100 and 1000 times this amount, in which confluent growth might have been expected, also yielded discrete and countable colonies. These colonies were considerably larger than the average. All the w.B.C. in stained smears of the heavily inoculated blood-organism mixtures contained large numbers of bacilli, an effect in part due to the high proportion of bacilli to w.B.C., and from these collections of living bacilli the large colonies presumably developed. The results in the more dilute mixtures showed that the phagocytosis was not lethal to any large degree; for, since the chance of an organism meeting a w.B.C. in unit time is the same in both mixtures there is no reason to suppose that the active phagocytosis deducible from the smears of the concentrated mixture did not operate in the mixtures containing only a few bacteria. The result is comparable with the “epiphylactic” phenomenon of Wright *et al.* (1923) but differs in that it was observed, not immediately after mixing blood and culture, but 2 hr. later. There is no necessity in this case, therefore, for postulating any cause other than the known one of aggregation in leucocytes.

Aggregation by agglutination. The circumstances favourable for this effect would be the presence of sufficient agglutinin to sensitize each bacterium rapidly without producing the “prozone” inhibition of excess antibody, and the absence of lethal or lytic effects dependent on specific antibody sensitization. The velocity of agglutination then depends on the concentration of bacteria.

We have no evidence to offer on staphylococci, but experiments with an organism of similar size, *Br. melitensis*, have shown that the time (*t*) of particulation with an optimally particulating concentration of antiserum is inversely proportional to the concentration, *C*, of the bacterial suspension, i.e. $t=K/C$. This relationship held when *C*, measured in terms of organism concentration, was reduced from 2×10^9 per c.c. to $10^9/4$ per c.c. Below values of $10^9/4$ per c.c., *K* increased with each reduction of *C*. It may be applied, nevertheless, with some confidence to a system like the dilute suspension of cocci in a bactericidal mixture, for the particulation time is likely to be much larger than that given by $t=K/C$ when *K* is determined for high concentrations of cocci. We shall therefore be over-estimating rather than underestimating the effect of agglutinins in producing aggregation. A 10^9 per c.c. suspension of *Staph. aureus* mixed with an optimal amount of antiserum is finely particulated into aggre-

gates of several hundred cocci in about 60 min. at 37° C. If we allow as little as 0.5 min. of the 60 for sensitization of the cocci and for the formation of aggregates of two cocci in such a mixture, then this degree of aggregation would occur in a concentration of 5×10^3 staphylococci per c.c. (i.e. that in a strong bactericidal mixture) only after $0.5 \times 10^9 / 5 \times 10^3$ min. = approx. 1500 hr. This estimate is a rough indication only of the magnitude of specific aggregation velocities, and we have no knowledge of factors which may operate in low concentrations. For example, it is possible that the rate may be increased with living flagellated bacteria, partly because their independent motion will increase chances of contact with each other, and partly because of their greater effective length and surface for sticking together after chance contact.

We have attempted to measure the effect of agglutinins in bacterium-antiserum mixtures, set up as bactericidal tests, compared with control normal serum-bacteria mixtures. Two systems were used—*H. parainfluenzae* and *Bact. coli*, with their respective complement-free rabbit antisera. In the tests on the first, the antisera were diluted in saline. In the *coli-anticoli* systems, the antisera were diluted in 1.0% heated normal rabbit serum, to obtain a constant concentration of serum proteins. Exps. 13–19 are summarized in Table VII, where the average numbers of bacteria in 0.02 c.c. of each serum-bacterium mixture are recorded. In the parainfluenza experiments, 13–16, the initial

Table VII. Mean colony counts in 0.02 c.c. of culture + homologous antiserum mixtures after 2 hr. rotation

Exp. ...	<i>H. parainfluenzae</i>						<i>Bact. coli</i>		
	(13)	(14)	(15b)	(15a)	(16b)	(16a)	(17)	(18)	(19)
Original inoculum	144	20	76	76	61	61	1	1	1
Control serum	34	15	17	31	15	12	140	137	157
Antiserum:									
1 in 24300	—	18	18	16	24	13	273	110	68
1 in 8100	—	17	25	33	54	25	83	128	233
1 in 2700	—	15	61	65	99	15	400	108	217
1 in 900	34	20	121	93	105	45	73	58	207
1 in 300	52	22	148	119	560	213	77	68	54
1 in 100	89	26	132	119	1073	800	19	18	10
1 in 33	130	—	—	—	—	—	—	—	—

number of viable organisms is reduced in the weak antiserum. The reduction is not due to agglutination, since it occurs in the control serum. With increasing strength of antiserum the count returns to the inoculum value, and exceeds it in the highest concentrations. Exps. 15 and 16 were modified in that after incubation two sets of samples were taken from the rotation tubes, (a) before and (b) after violent agitation with a pipette.

Forcible agitation in 15 increased the strong serum counts, but not significantly. In 16 there was a significant increase, especially marked in counts equal to or greater than the inoculum, which suggests that disintegrable bacterial clumps are formed in the mixtures containing multiplying organisms. The *H. parainfluenzae* formed granular masses in broth cultures, and the

clumps may have been incompletely separated daughter cells. On the other hand, daughter cells may have failed to separate into single colony-forming units because they were sensitized by antibody, and so multiplied in agglutinated masses. The *Bact. coli* experiments, 17-19, support this view. The serum protein content was constant in all mixtures, and the strain of *coli* grew fully dispersed in fluid cultures, i.e. the daughter cells had no inherent tendency to stick together. In all three experiments the growth was so rapid that countable numbers of colonies were produced only in serum-bacterium mixtures initially seeded with 50 organisms per c.c. In 18, the growth was greatest in the control, and least in concentrated serum. In 17 and 19 there was an initial increase with increasing antibody concentration, followed by a marked decrease. The decrease may have been due to a genuine lethal action of the antiserum; but if agglutination was the cause, the organisms were in all probability agglutinated as they divided, since even in the strongest antibody solution there was 10-20-fold increase of the original inoculum. It is unlikely that the increase represents the growth of a few free organisms not included in an initially agglutinated mass. This might happen if the available agglutinins were completely absorbed by a proportion of the bacteria. But the agglutinin content of the two sera was well in excess of the amount required to sensitize the maximum number of bacilli counted; the titres were 1 : 640 and 1 : 5120 respectively, i.e. concentrations capable of agglutinating 10^9 organisms per c.c.

The results suggest that specific agglutinins can exert an apparently bacteriostatic action on multiplying organisms by keeping the daughter cells in clumps, but there is no evidence of direct clumping of the small numbers of organisms *initially dispersed* in the antiserum, in the 2 hr. rotation period of the bactericidal tests.

It is open to question whether similar results would be obtained if the experiments could be made in blood-bacterium mixtures. The initiation of agglutination and phagocytosis depends on close approximations of one bacterium with another, or with a w.b.c., and the presence of 5×10^9 R.B.C. per c.c. may interfere with such approximations. Two particles in a fluid move towards one another as a result either of Brownian movement or shearing distortions of the particular piece of fluid that contains them. A body of fluid occupying the position of a R.B.C. between two cocci a few microns apart would allow the approximation of the cocci should they be driven together by chance molecular bombardments, but an actual R.B.C. in this position would interfere with it and, by reason of its inertia and size, would tend to remain in the interfering position. On the other hand, R.B.C.'s would interfere very little with approximations due to the shearing movements of a well-agitated fluid; they would behave as particles of fluid of the size and volume of a red cell, round which the actual fluid would slide with ease. It seems not unlikely that the presence of R.B.C.'s reduces the rate of agglutination and phagocytosis in blood-bacterium mixtures by a small and as yet undetermined amount depending on the relative importance of Brownian and mixing movements in securing approximation.

Further evidence on these points is needed. In its absence, it seems reasonable to assume that, in mixtures containing small inocula, fallaciously large degrees of bactericidal power are unlikely to occur as the result of mechanical

aggregation of colony forming units. Agglutination and non-lethal phagocytosis may reduce the colony counts of large inocula. The bactericidal test described allows an adequate check on such effects. If the reduction of a large inoculum exceeds that of the corresponding inoculum one-tenth or one-hundredth its size, i.e. if p decreases with increasing inoculum, then hypotheses, e.g., of mechanical aggregation and of epithylactic responses must be considered. In our experiments, the two values of p calculated from mixtures made with adjacent culture-dilutions of the 10-fold series never differed significantly. From 40 unselected determinations of normal values of p made from inocula between 5 and 8, and from the corresponding inoculum of 50–80, the average difference was 0.05, a value equal only to one-half the standard error of the distribution of normal values.

NORMAL AND ABNORMAL BACTERICIDAL POWER

In Table VIII A are listed the results of tests on the blood of healthy and of infected adults, with the strains P 802 and P 900. There are five groups of values for p : group I, those of eleven healthy adults tested with P 900; group II, those of six healthy adults tested with P 802; group III, those of nine determinations on the blood of one healthy adult, at intervals in a period of ten weeks; group IV, those of seven persons with chronic staphylococcal infections, some of them under treatment with staphylococcal toxoid; and group V, those of two persons with acute *Streptococcus pyogenes* cellulitis.

In Table VIII B are recorded the differences between various selected pairs of results within the first four groups, expressed in terms of the standard error of the differences (i.e. the ratio d/σ_d). If the ratio is in excess of 3.0, the two different values of p concerned cannot be said to have arisen by chance from tests on bloods of essentially the same bactericidal power. The demonstration of significant differences is therefore evidence of the heterogeneity of the bloods tested, as far as their bactericidal power is concerned.

The differences between extreme values, and between extreme values and those lying closest to the average value of p for the group, have been calculated. For example, tests 7 and 8 are nearest to the mean 0.143 of group I, and test 27 a to the mean 0.0331 of group IV. In group III all the first eight values differ significantly from the last (test 26). This outstanding value of 0.422 was determined in blood taken after a day's "malaise and insomnia" (recorded before the result of the test was known), and may be excluded as a possibly abnormal value. The extremes (18 and 25) of the remainder differ significantly, i.e. the bactericidal power of the blood of one person may undergo significant fluctuations, and until the limits of fluctuations of p for a healthy person are known, the inclusion of a single "normal" blood in a series of bactericidal tests, as a means of fixing "normal" bactericidal power for a given experiment, is an unreliable procedure.

The majority of selected pairs tested in each group yield ratios > 3 , and

Table VIII A. Values of p ; the blood of adult human beings

No. of test	Source of blood	Group; and strain of <i>Staphylococcus</i> used	p	σ_p
1	17 healthy adults	Group I (P 900)	0.061	0.0000879
2			0.062	0.0001174
3			0.070	0.0001592
4			0.087	0.0001687
5			0.108	0.0002543
6			0.114	0.0001726
7			0.125	0.0002130
8			0.166	0.0002932
9			0.168	0.0002667
10			0.219	0.0005675
11			0.294	0.0005764
12	Group II (P 802)	0.100	0.0001198	
13		0.139	0.0001724	
14		0.164	0.0001944	
15		0.168	0.0001999	
16		0.252	0.0003214	
17	0.269	0.0003718		
18	Nine specimens from one healthy adult, during a 10-week period	Group III (P 802)	0.080	0.0001406
19			0.104	0.0002422
20			0.139	0.0001510
21			0.144	0.0002018
22			0.156	0.0001747
23			0.164	0.0001944
24			0.182	0.0002445
25			0.197	0.0003037
26			0.422	0.0005392
27 b	Patients with <i>Staph. aureus</i> infection	Group IV (P 900)	0.0143	0.000003929
27 c			0.0158	0.000005064
28			0.0231	0.000006805
29			0.0262	0.000008175
30			0.0280	0.000008274
31 a			0.0305	0.000009292
27 a			0.0315	0.000009457
32			0.0350	0.000013054
33	0.0591	0.00007526		
31 b	0.0665	0.00003258		
34	Patients with <i>Str.</i> <i>pyogenes</i> cellulitis	Group V (P 900)	0.434	0.0007483
35			0.357	0.0005825

Table VIII B. The ratio d/σ_d for differences between the values of p in selected pairs of tests from Table VIII A

Group	Tests	d/σ_d
I	1 and 11	9.03
	1 and 7	3.69
	1 and 8	5.38
	7 and 11	6.01
	8 and 11	4.34
II	12 and 17	4.33
III	18 and 25	5.57
IV	27 b and 31 b	5.40
	27 b and 27 a	4.70
	27 a and 31 b	2.88
	27 a and 27 b	4.70
	27 a and 27 c	3.88
	31 a and 31 b	5.41

the values of p represent a series of individual responses to *Staph. aureus*. The wide variation is not due to day-to-day changes in experimental conditions, for significantly different values were obtained from bloods tested at one time with the same culture dilutions, batch of medium, etc. The number of tests is small, but there are indications that the lower limit of p , in healthy adults not recently convalescent from staphylococcal infections, may prove to be in the region of 0.05 for *Staph. aureus* strains similar to those used in the above tests.

The blood of *Staphylococcus*-infected patients undergoes changes during the course of the disease. Blood 27 (group IV) from a patient with chronic osteomyelitis, was tested on three occasions: (a) 5 months after a first course of toxoid, and immediately prior to a second course, (b) and (c), 1 and 4 weeks after the second course had been started. 27b and c are both significantly different from a, though not from each other.

31a and b, taken respectively in the active and convalescent stages of a staphylococcal breast abscess, differ by 0.035, which is 5.41 times its standard error.

The two "streptococcal" values in group V (34 and 35) are well above the mean value for normal bloods.

SUMMARY

The survival rate, p , of a measured inoculum of *Staph. aureus* in a standard volume of defibrinated blood, is a reliable quantitative measure of the bactericidal power of blood. The number of viable organisms in the inoculum and in the blood-bacterium mixture may be estimated with the necessary accuracy by counts of colonies developing from measured volumes of the fluids let fall on to the surface of solid media. Fildes' agar was the most suitable medium for this surface-viable count, and was selected on the basis of four criteria; of the media tested it yielded the highest counts, and the counts conformed most closely to a Poisson series; and on it the mean colony size was maximum, and the coefficient of variation of colony size was minimum. On this medium, the close conformity of the separate count values to a Poisson series enabled the standard error of the survival rate to be determined from a simplification of the general expression for the standard error of a ratio.

The number of colonies growing from a sample of a blood-bacterium mixture may be reduced, not by killing of the individual cocci, but as a result of their aggregation either by agglutinins in the blood, or in the cytoplasm of leucocytes that are phagocytic but not bactericidal. It appears that these mechanisms are unlikely to operate in blood-bacterium mixture containing relatively few organisms; in such mixtures the survival rate is a reflexion of the killing power only.

The immunological significance of p has not been investigated, but the range of values for healthy human adults differs significantly from that for sufferers from chronic staphylococcal infection. Moreover, by the technique

employed differences may be detected between individual values of p that cannot reasonably be attributed to technical or sampling errors.

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A NOTE ON THE STANDARD ERROR OF THE SURVIVAL RATE OF
 BACTERIA IN BLOOD-BACTERIUM MIXTURES

BY J. O. IRWIN

n bacteria are added to the blood, and r survive. r and n are derived from the mean of N plates, and are subject to error.

The survival rate $p = r/n$.

Taking logarithmic differentials

$$\frac{\delta p}{p} = \frac{\delta r}{r} - \frac{\delta n}{n},$$

whence, writing $V(n)$ for the sampling variance of n , etc.,

$$\frac{V(p)}{p^2} = \frac{V(r)}{r^2} + \frac{V(n)}{n^2},$$

$$V(p) = \frac{p^2}{r^2} V(r) + \frac{p^2}{n^2} V(n),$$

$$V(r) = npq/N,$$

and

$$V(p) = \frac{pq}{nN} + \frac{p^2}{n^2} V(n),$$

i.e.

$$\sigma_p^2 = pq/nN + p^2\sigma_n^2/n^2.$$

In the tests described above, $n = km$, k being a constant depending on the dilution of culture, and m the mean number of colonies counted. If m is the mean of a Poisson series, we have

$$\begin{aligned}\sigma_n^2 &= \frac{k^2 m}{N} \\ &= \frac{n^2}{mN}.\end{aligned}$$

Hence

$$\begin{aligned}\sigma_p &= \sqrt{\left(\frac{pq}{nN} + \frac{p^2}{mN}\right)} \\ &= \sqrt{[p + (k-1)p^2]/nN}.\end{aligned}$$

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