SOME OBSERVATIONS ON THE RIDEAL-WALKER TEST

By A. C. THAYSEN
Microbiological Section, Chemical Research Laboratory, Teddington, Middlesex

Discrepancies in coefficient values of the order of those recorded by the Laboratory of the Royal Institute of Public Health (1923) were obtained by the writer when determining the Rideal-Walker coefficient of four samples of disinfectants. In these determinations he had adhered strictly to the technique laid down in the British Standards Institution's specification No. 541, of 1934.

In the publication of the Royal Institute of Public Health the irregularities found were thought to have been caused partly by the influence of temperature, media, size of drops transferred and by the phenol samples used, in other words by practically every link in the Rideal-Walker technique. However, since all of these causes of variation had been fully considered by, and had been eliminated from, the technique as laid down in the B.S.I. specification, it seemed probable that the writer's difficulties were due to other sources of error, particularly since they were met with even in the preliminary phenol tests which are required for the calculation of the coefficient value of the disinfectants to be tested.

The three experiments quoted in Table I show that even when adhering strictly to the technique of the B.S.I. specification it was impossible to guarantee uniform results in the phenol tests.

Table I

Phenol concentration	Exposure of the test culture (0·2 ml.)			
	2½ mins.	5 mins.	7½ mins.	10 mins.
1 : 95	+	+	−	−
1 : 100	+	+	−	−
1 : 105	+	+	+	−
1 : 110	+	+	+	+
1 : 115	+	+	+	+
1 : 95	+	−	−	−
1 : 100	+	+	+	−
1 : 105	+	+	+	−
1 : 110	+	+	+	−
1 : 115	+	+	+	+
1 : 95	+	−	−	−
1 : 100	+	+	+	−
1 : 105	+	+	+	−
1 : 110	+	+	+	−
1 : 115	+	+	+	+

In two of these experiments no stage occurred in which a given phenol concentration required more than 5 min. and less than 7½ min. to destroy the

https://doi.org/10.1017/S0022172400011414 Published online by Cambridge University Press

added bacteria. Since the existing specification does not provide for the interpretation of such contingencies it was felt that lack of experience in carrying out the test was primarily responsible and that greater uniformity would be obtainable when greater skill had been acquired. Advice was therefore sought and received from an acknowledged expert on the Rideal-Walker technique, and as a result the writer's method of carrying out the test was modified in two respects. However, the very considerable amount of work with the test which was done subsequently did not show any significant improvement in the uniformity of results obtained, but gave the impression that the difficulty of securing reproducible results, even in the phenol tests, was due not to the failure of maintaining a uniform standard of technique but to some biological factor in the cells exposed to the action of the disinfectant.

In forming this opinion it was not overlooked that several investigators of the action of disinfectants have deduced from their results that the reaction between bacteria and disinfectant conforms to the law of mass action, a conclusion which, if correct, should make it possible to obtain reproducible results. Unfortunately, however, it is generally admitted (Chick, 1930) that any uniformity with the law which the reaction between disinfectant and bacteria may show applies to that period of exposure only during which the bulk of the test cells is being destroyed. It is recognized that there is present in every bacterial population a small number of cells which will survive the action of a disinfectant very much longer than the bulk.

It was obviously desirable to find out whether the writer's failure to secure uniform results in the Rideal-Walker test might have some connexion with the presence in his test culture of a small percentage of highly resistant cells.

To establish this it was necessary to amend the standard technique of the test so as to be able to count the actual number of surviving cells transferred from the solutions of disinfectant to the growth tubes. To do this 1·5% of agar was added to the broth in the growth tubes. This necessitated maintaining the tubes at 45° C. while they were being mixed, in Petri dishes, with a standard loopful of the various exposed mixtures of phenol solution and test organisms.

In one experiment with phenol, carried out in this way, the result shown in Table II was obtained. By appropriate counts it had been ascertained that

Table II

Concentration of phenol solution	Number of test organisms surviving exposure for			
	2½ mins.	5 mins.	7½ mins.	10 mins.
1 : 95	90	2	0	0
1 : 100	273	10	7	1
1 : 105	230	53	6	3
1 : 110	1760	139	117	0
1 : 115	approx. 6000	330	103	89

the 0·2 ml. of culture used to inoculate each phenol solution had contained 480 million living cells, so that one standard loopful of inoculated phenol solution would have contained 1·34 million cells. It is, therefore, an extremely

small percentage of this total which in some cases has survived the action of the disinfectant and which has changed an almost normal rate of destruction into quite an exceptional survival, at least when the results are expressed in the usual way of the Rideal-Walker technique with a plus sign for the survival even of one single cell and a minus sign only when every cell has been destroyed.

The experiment was repeated several times with results which, though by no means identical with that quoted in Table II, did at least bear out that any observed deviations from the expected smooth curve of survival were due to the resistance of a very small number of the cells originally introduced into the phenol solutions. It was clear also from these experiments that the survival must have been due to causes other than the partial destruction of the phenol contained in the solutions through its reaction with the protein of the added bacterial cells, for the amount of phenol in each of the solutions used, even in the most dilute, was more than a thousand times greater than the weight of the cells added, if Nägeli's figure of $1/10^{-10}$ mg. is accepted as the weight of an average bacterium (Frost & McCampbell, 1915). With such an excess of disinfectant over test organisms the rate of destruction should not, according to Buchanan & Fulmer (1930), be influenced by a possible partial destruction of the phenol by the proteins of the added cells.

Having found that the observed irregularities were due to the survival of a very small fraction of the number of cells originally exposed to the disinfectant, the question naturally arose whether this survival could be due to a greater resistance than that normally exhibited in a small but definite fraction of the whole bacterial population. It was argued that if it was, it should be possible, by a reduction in the number of cells added to the disinfectant, to reduce the number of cases in which unexpected survival occurred, for such cases were often due, as has been shown, to the resistance of a few, or even of one single cell.

In Table III is recorded the result of three experiments in which the number of cells added to the disinfectant had been reduced below the standard number. Of the three, the first is directly comparable with the experiment in Table II, for it was carried out on the same day, with the same culture and under identical conditions as regards technique as the latter, with the exception that only half the original number of cells was added.

These experiments lend little support to the view that the surviving cells of a 24 hr. old culture represent a definite proportion of exceptionally resistant cells of the total bacterial population, though it must be admitted that the first series of Table III, which is directly comparable with that of Table II, showed a remarkably uniform survival rate. But a repeat series failed to confirm this improvement, which had been achieved by a reduction in the number of exposed cells, and the further halving of the number of cells (as in series 3 of Table III) did most decidedly not increase the regularity of the survival.

Table III

Concentration of phenol solution	Number of test organisms surviving exposures for			
	2½ mins.	5 mins.	7½ mins.	10 mins.
1 : 95	11	0	0	0
1 : 100	26	4	0	0
1 : 105	134	6	3	0
1 : 110	186	56	27	12
1 : 115	608	98	12	3
Amount of culture (24 hr. old) added to the phenol solution = 0·1 ml., equal to 240×10^6 cells. Number of cells per standard loopful of inoculated phenol solution = 674,000.				
1 : 95	12	0	0	0
1 : 100	13	1	0	0
1 : 105	101	6	1	2
1 : 110	424	41	26	4
1 : 115	660	115	38	31
Amount of culture (24 hr. old) added to the phenol solution = 0·1 ml., equal to 240×10^6 cells. Number of cells per standard loopful of inoculated phenol solution = 674,000.				
1 : 95	1	1	0	0
1 : 100	22	0	0	0
1 : 105	56	5	1	0
1 : 110	80	27	9	1
1 : 115	126	24	14	0
Amount of culture (24 hr. old) added to the phenol solution = 0·05 ml., equal to 120×10^6 cells. Number of cells per standard loopful of inoculated phenol solution = 337,000.				

Though the reason for survival and for resultant irregularity in the test were thus more complex than might be explained by the assumption of the existence in the population of the test culture of a definite fraction of highly resistant cells, the fact remained that a small percentage of cells in the test culture behaved differently from the bulk. Since it has been pointed out (Chick, 1930) that a 24 hr. old culture may not necessarily represent an age at which all the cells of a culture are most uniform in character, but that the desirable requirement of maximum uniformity may be more readily secured by the use of cultures which are but a few hours old and in a state of active growth, a series of experiments was carried out in which the behaviour was studied of cultures of *Bact. typhosum* grown at 37° C. for 4, 5 and 6 hr.

It will be seen from Table IV that there is nothing in the experiments there recorded which suggests that the use of a very young culture may lead to greater uniformity in the Rideal-Walker test. Further, neither these nor any of the earlier experiments reveal a tangible explanation for the irregularities to which attention has been drawn in these pages, though they do establish that the irregularities are caused by the survival of a very few cells of the bacterial populations used in the test.

As it had been suggested that *para*-chlor-*meta*-cresol possessed advantages over phenol as a control disinfectant, several experiments were carried out to find out whether greater uniformity in survival rate could be obtained by the use of this substance than of phenol. This, however, was not found to be so. Without quoting actual figures it can be claimed that more often than not it

Table IV

Concentration of phenol solution	Number of test organisms surviving after exposure for			
	2½ mins.	5 mins.	7½ mins.	10 mins.
1 : 95	2	0	0	0
1 : 100	9	0	0	0
1 : 105	31	2	0	0
1 : 110	25	2	0	0
1 : 115	89	26	5	0

Amount of culture (4 hr. old) added to the phenol solution = 0·2 ml., equal to 24×10^6 cells.
Number of cells per standard loopful of inoculated phenol solution = 67,600.

1 : 95	1	0	0	0
1 : 100	3	1	1	0
1 : 105	56	2	1	1
1 : 110	166	12	1	0
1 : 115	243	28	0	1

Amount of culture (5 hr. old) added to the phenol solution = 0·2 ml., equal to 56×10^6 cells.
Number of cells per standard loopful of inoculated phenol solution = $0{\cdot}16 \times 10^6$.

1 : 95	67	3	1	0
1 : 100	171	21	4	5
1 : 105	512	10	55	7
1 : 110	776	280	78	47
1 : 115	1032	608	92	60

Amount of culture (4 hr. old) added to the phenol solution = 0·2 ml., equal to 66×10^6 cells.
Number of cells per standard loopful of inoculated phenol solution = $0{\cdot}19 \times 10^6$.

Two repeat tests with a 6 hr. old culture gave the following results:

1 : 95	0	0	0	0
1 : 100	43	3	0	0
1 : 105	154	12	3	0
1 : 110	364	85	25	3
1 : 115	2240	156	48	8

Amount of culture (6 hr. old) added to the phenol solution = 0·2 ml., equal to 96×10^6 cells.
Number of cells per standard loopful of inoculated phenol solution = $0{\cdot}27 \times 10^6$.

1 : 95	21	0	0	0
1 : 100	52	2	1	0
1 : 105	103	10	3	1
1 : 110	336	46	10	4
1 : 115	312	89	38	0

Amount of culture (6 hr. old) added to the phenol solution = 0·2 ml., equal to 62×10^6 cells.
Number of cells per standard loopful of inoculated phenol solution = $0{\cdot}17 \times 10^6$.

was impossible to obtain the uniform rate of destruction which is required by the Rideal-Walker test and that where irregularities were met with, they were invariably due to the abnormal survival of a very few cells. Further, it should be added that both in the case of *para*-chlor-*meta*-cresol and of phenol the irregularities to which attention has been drawn occurred with all the strains of *Bact. typhosum* which were tested, both with those obtained from the National Collection of Type Cultures and with a strain secured from the acknowledged expert on the Rideal-Walker test to whom reference was made earlier in these pages.

So far the results of the observations made showed that no technique could be devised by the application of which the resistance of all the cells of a test culture exposed to the action of an antiseptic could be brought to conform

to a definite standard rate of destruction. It still remained to be determined whether the observed variability in resistance of the cells could be counteracted by increasing the time during which the cells were left exposed to the action of the antiseptic.

In the series quoted below the exposure of the cells was continued for 30 min. instead of 10, and every 30 sec. during this time one standard loopful was transferred from each inoculated phenol solution to 5 ml. of standard broth solidified with agar.

Table V

Concentration of phenol solution	Number of test organisms surviving after exposure for											
	2½ mins.	5 mins.	7½ mins.	10 mins.	12½ mins.	15 mins.	17½ mins.	20 mins.	22½ mins.	25 mins.	27½ mins.	30 mins.
1 : 95	14	0	0	0	0	0	0	0	0	0	0	0
1 : 100	68	1	3	3	0	0	0	0	0	0	0	0
1 : 105	56	19	8	2	0	1	0	0	0	0	0	0
1 : 110	1080	138	58	5	1	0	0	0	0	0	0	1
1 : 115	2880	125	28	43	4	1	0	0	0	0	0	0

The results are of considerable interest. It will be seen that with one exception the standard loopfuls of inoculated phenol solutions contained no living organisms after 17½ min. Prior to that occasional survivals were recorded, survivals which, however, were too irregular to suggest an extended period of exposure as a remedy for overcoming the difficulties inherent in the Rideal-Walker technique. But the interest of the experiment is to be sought elsewhere than in its support for this conclusion.

For, if it may be assumed that the one survival recorded after 30 min. exposure was not accidental, then it must be concluded that even after the prolonged exposure of 30 min. there remained living cells in the inoculated phenol solutions or at least in one of them. The number of these cells, however, must have been so small that only occasionally would one of them be successfully caught in the small quantity of liquid which is transferred by the standard loop, even when the various inoculated phenol solutions are vigorously shaken before transfer, as was invariably the case in the experiment of Table V.

To test this point much larger quantities of the inoculated phenol solutions were transferred after 30 min. exposure of the cells to equally increased quantities of standard broth. In the actual experiments 2·8 ml. of inoculated phenol solution, that is 200 times the normal quantity, were transferred to 1 l. samples of standard broth. The result obtained was not unexpected. The data recorded are given in Table VI.

This can only be interpreted as showing that the Rideal-Walker test, when it allots a certain coefficient value to an antiseptic, fails to insure that *all* the cells of a culture of *Bact. typhosum* added to the antiseptic will have been destroyed after exposure for the appropriate time.

Where, as shown in Table V, the standard time of exposure is extended beyond the 10 min. specified in the Rideal-Walker test it will become in-

Table VI

Concentration of phenol solution	After exposure in phenol solution for 30 sec. growth of *Bact. typhosum* was obtained in broth after	
	24 hr.	48 hr.
1 : 95	Nil	+ + +
1 : 100	+	+ + +
1 : 105	+ + +	+ + +
1 : 110	+ + +	+ + +
1 : 115	+ + +	+ + +

+ indicates slight but definite growth. + + + indicates abundant growth.

creasingly difficult to secure living cells in the small standard quantity of liquid which is transferred to the growth tubes (0·014 ml.) until after 17–18 min. with phenol, it will in most cases be impossible to do so. This implies that any test, the Chick-Martin test, for instance, which is based on an exposure of the test organism to the antiseptic for 30 min., is much more likely to yield consistent results than the Rideal-Walker test. But neither the Chick-Martin nor the Rideal-Walker test must be taken to guarantee the complete destruction of the exposed cells within the exposure time stipulated. It follows that if it is desirable, which it obviously must be, to know when complete destruction of all the cells exposed to an antiseptic has been secured, it is necessary to transfer all, or at any rate the bulk of the exposed cells to favourable growth conditions, as was done in the experiment quoted in Table VI. This was tried in a further series of experiments in which it was established that an exposure to a phenol solution of the dilution 1 : 95 under standard conditions for 45 sec. had destroyed all the added cells of *Bact. typhosum*. The actual results obtained in this series of experiments are tabulated in Table VII.

Table VII

Time of exposure at 17·5° C.	Concentration of phenol solution inoculated with 0·2 ml. 24 hr. old culture of *Bact. typhosum*					
	1 : 95	1 : 100	1 : 105	1 : 110	1 : 115	Remarks
30 min.	+ + +	+ + +	+ + +	+ + +	+ + +	Growth estimated after 48 hr. at 37°
45 min.	0	+ + +	+ + +	+ + +	+ + +	
60 min.	0	0	0	0	0	

In each case 2·8 ml. of the inoculated phenol solution were added to 1 l. of standard broth after the exposure indicated. The experiment was repeated with identical results in the case of the dilutions 1 : 95 and 1 : 115 with the whole of the 5 ml. of inoculated phenol solutions, transferred after exposure for 45 respectively 60 min. to 2 l. of standard broth. It can be concluded, therefore, that an exposure of 45 min. to a phenol solution of 1 : 95 strength and 60 min. for the remaining dilutions are sufficient to destroy all the added cells of a culture of *Bact. typhosum*.

The practical significance of this observation would appear to be that a coefficient value for an antiseptic, determined by the Chick-Martin test, may

be expected to secure complete destruction of all the cells of *Bact. typhosum* added to the antiseptic, within double the time used in the actual experiment for the determination of the coefficient value unless, of course, it must be assumed that some antiseptics have a slower rate of functioning than phenol. In such cases it would be necessary to carry out an experiment with the antiseptic in question on the lines suggested in Table VII before expressing an opinion as to the true efficiency of the antiseptic.

CONCLUSIONS

Attention has been drawn to the difficulty of obtaining reproducible results by the Rideal-Walker test for the evaluation of antiseptics.

These difficulties have been shown to arise from the survival of a few cells of a bacterial population when exposed to the action of the antiseptic tested.

The surviving cells are present in the inoculated solutions of the antiseptics undergoing tests in numbers which in time become too small to ensure that one cell at least will be transferred to the growth tubes with the very small proportion of the whole inoculated antiseptic solution taken. After an exposure of the test organism for 17–18 min. in phenol solutions the surviving cells are so few that it is practically impossible to detect their presence in the growth tubes except by the transfer of much larger quantities of inoculum than required by the test.

These observations imply that a method such as the Chick-Martin test, which is based on the exposure of the test cells for 30 min. will yield far more consistent results than the Rideal-Walker test with its 10 min. exposure of the test cells.

Neither of the tests, however, must be interpreted as expressing that a given coefficient value implies that an antiseptic utilized on the basis of this value will completely destroy the test organism during the time interval stipulated by the test.

To secure complete destruction of *Bact. typhosum* in phenol solutions a 1 hr. exposure of the test organism will generally be necessary.

ACKNOWLEDGEMENT. This communication is published with the permission of the Director of Chemical Research in the Department of Scientific and Industrial Research.

REFERENCES

BUCHANAN, R. G. & FULMER, E. J. (1930). *Physiology and Biochemistry of Bacteria*, **2**. London: Baillière, Tindall and Cox.

CHICK, H. (1930). *A System of Bacteriology*, **1**, 179. H.M. Stationery Office.

FROST, W. D. & MCCAMPBELL, E. F. (1915). *A Text-Book of General Bacteriology*. New York: Macmillan.

LABORATORY OF THE ROYAL INSTITUTE OF PUBLIC HEALTH (1923). *J. State Med.* **31**, 477.

(*MS. received for publication* 2. IV. 1938.—Ed.)