THE TESTING OF DISINFECTANTS IN THE PRESENCE OF ORGANIC MATTER

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(With 1 Figure in the Text)

IT is now almost universally recognised that a test for disinfectants which is to yield results of direct practical significance must include appropriate "organic matter" in the test mixture. Some types of disinfectant, notably those which act by liberating chlorine or oxygen, are greatly reduced in activity by the addition of only small quantities of any extraneous material with which these elements can combine. Other disinfectants are more resistant to this influence; they are capable of efficient action in the presence of excreta and dirt of other kinds if used in adequate concentration. What this concentration should be under such unfavourable conditions it is necessary to determine, since the degree of interference is not the same for all disinfectants even in the same class, and no constant relationship exists between efficiency under these conditions and that displayed in a medium of distilled water as determined by the Rideal-Walker test. Numerous efforts have been made in the past to introduce tests employing adequate amounts of complex organic matter; few of these have achieved permanence and none popularity. The following short survey of past work is concerned only with what may be termed "excremental" disinfectants; the testing of surgical disinfectants, which may reasonably be held to demand different methods, is outside the scope of this paper.

PREVIOUS TESTS EMPLOYING ADDED ORGANIC MATTER

The history of modern disinfectant testing dates from 1903, when the older thread and garnet methods were displaced by one depending on absolute sterilisation in a purely fluid mixture. The Rideal-Walker method is the prototype of several widely used present-day tests, and its principle is one which is likely always to be employed in some form. The widespread adoption of the original Rideal-Walker method and the quotation of numerical coefficients based on it regardless of their limited significance resulted in a series of protests, and in attempts to introduce tests employing added organic matter. These proposed tests were of two kinds, those in which highly complex materials were used, such as faeces, urine and milk, and those employing comparatively simple additions such as gelatin and starch. From each of these

suggested modifications there emerged eventually a test which is still in use at the present day.

The earlier advocates of excreta as added organic matter described methods which appear exceedingly crude by modern standards, and it is not surprising that another section of opinion vigorously opposed their adoption. Fowler (1906) employed a 1 per cent. suspension of faeces in urine, which was filtered to remove coarse particles; the test was performed with a mixture of equal volumes of this suspension and dilutions of the disinfectant. This addition had apparently little effect on the activity of coal-tar disinfectants; it greatly diminished that of mercury perchloride, potassium permanganate and iodine, as would be expected. Firth and MacFadyean (1906) recommend the same method, while Kenwood and Hewlett (1906) suggest the even cruder proceeding of adding a 5 per cent. disinfectant solution in different amounts to natural liquid faeces and observing the volume required to destroy *B. coli* in half an hour. Winter Blyth (1906*a*) found surprising reductions in the activity of coal-tar disinfectants when milk was added to the test mixture, and subsequently (1906*b*) reported an extensive series of experiments with both milk and faeces, in which the chemical composition of these additions was studied in considerable detail, and the faeces were dried, ground and weighed in the dry state before addition to test mixtures.

These proposals, even including the last, which had at least some pretensions to accuracy, were not only resisted but ridiculed by other workers, whose contention was that natural excreta and secretions are too variable in physical characters and chemical composition for any test employing them to give uniform results. Sommerville and Walker (1906, 1907), who took the chief part in this protest, performed a series of tests in which the organic matter consisted of 1 per cent. of gelatin, casein, peptone, mucin, serum or blood; these additions reduced the activity of potassium permanganate and a hypochlorite solution to a small fraction of that exhibited in distilled water, but affected that of coal-tar disinfectants comparatively little. If all that is required of such a test is that it should indicate the limitations of oxidising and chlorinating disinfectants, such simple and scanty additions as these will doubtless serve; it has even been suggested much more recently (Reddish, 1927) that the requirements for organic matter be met by using 0·5 c.c. of culture as the inoculum for 5 c.c. of disinfectant solution instead of the smaller volume more commonly employed. In order to furnish particulate as well as dissolved organic matter and consequently to provide for the possibility of adsorption of the disinfectant, Sommerville and Walker (1908) subsequently proposed a method in which the diluent contained 0·5 per cent. of gelatin and 0·5 per cent. of rice starch; this gave results closely comparable to those of their previous methods. The present Admiralty test (Patterson and Frederick, 1931) appears to have been developed from this method, since it employs the same organic additions in the same concentration; it differs in the fact that the diluent is artificial sea-water and in numerous details of technique.

While the Admiralty test was the only eventual result of the work just described, the culminating point of studies of disinfection in the presence of excreta was the publication of a proposed method of test by Chick and Martin (1908) which is still known by their names and remains in this country the only recognised method of general application for testing disinfectants in the presence of organic matter.

THE CHICK-MARTIN TEST

The Chick-Martin test was in an entirely different category from the crude proceedings with excreta which had previously been recommended. It was based on an exhaustive study of the mechanism of disinfection under varying conditions, and it exhibited neither of the gross and obvious defects of most of these previous methods; the bacteria in the faeces employed were destroyed, the test organism being added in the form of a measured volume of culture, and one variable factor at least in the composition of faeces, its water content, was overcome by previous drying. The method is briefly as follows. Normal human faeces are dried, ground in a mortar, and passed through a sieve with a mesh of 130 to the inch. The resulting fine powder is weighed in quantities of 0·15 g. and these are placed in tubes to which 2·5 c.c. of distilled water are added; these are then autoclaved. Quantities of diluted disinfectant and distilled water together totalling a further 2·5 c.c. are added to these tubes, which thus come to contain 3 per cent. of dried faeces and varying concentrations of the disinfectant. Similar dilutions of phenol are prepared without an addition of dried faeces. The tubes are kept at 20° C., 0·1 c.c. of *B. typhosus* culture is added, and one loopful of the mixture is cultivated after 30 minutes. These are the main features of the test; in a number of details, such as the method of preparing dilutions, the culture media used, and the calculation of the coefficient, it differs from other methods.

It is a singular fact that since Chick and Martin's original description, no further information about this test, whether in the form of results obtained with it or suggestions for its improvement, was published during a period of twenty-six years, although during that time the test was not only the only available one of its kind, but secured a considerable measure of official recognition. A partial explanation of this fact is that the Chick-Martin test has been little used except in circumstances where its official status compels its application. The unpopularity of this test has been due to two principal causes. Its performance is offensive and tedious, the former because the process of grinding and sieving dried faeces causes aerial dispersion of many of the finer particles, the latter both on account of this same process and owing to the necessity of weighing the dried faeces for addition to each individual tube. These objections could not be considered insuperable if they were inevitable, but a more serious criticism has been that the test does not give consistent results. "Irregular tubes" are common, and it is considered advisable to base a coefficient only

on the average of several tests, the individual results of which may vary considerably. Coefficients obtained even in this way cannot always be confirmed by careful workers employing the same technique in other laboratories. These facts suggest that the test has inherent faults which render accurate findings impossible, and evidence has been given in a previous paper (Garrod, 1934) of the existence of such faults. The sources of error demonstrated depend upon the physical characters and behaviour of the suspension of faeces. This suspension consists mainly of comparatively large particles, and these can be disintegrated by shaking; the greater the degree of this disintegration the greater was the interference with the action of a disinfectant. The amount of shaking (a necessary part of the test, and the extent of which obviously cannot be prescribed in exact terms) may therefore affect the result of a test, as may any other factor affecting the size of the particles of faeces; the vigour with which grinding is carried out is one of several other possible factors having this effect. The second source of possible error demonstrated is the rapid formation of a compact deposit of faecal particles in which small numbers of typhoid bacilli survive when the concentrations of disinfectant is sufficient rapidly to sterilise the supernatant fluid. Not only will the result given by such a tube depend entirely on whether the inoculum removed contains some of this deposit, but even assuming that it does, the number of surviving bacilli is apt to be so small that chance plays too large a part in determining the result. To these demonstrated defects may be added several theoretical objections of which the practical consequence is undetermined; these are the admixture of disinfectant and organic matter before the addition of culture, which allows adsorption to proceed for an unspecified period before disinfection begins, the absence of organic matter from the phenol tubes, the dispersion of faecal particles on the walls of the tube above the fluid level, the uncorrected reaction of the faecal suspension (found in the above experiments to be distinctly acid), and variations in the general composition of the faeces used. Although the original work of Chick and Martin includes experiments which suggest that different samples of faeces give similar results, the possible variations in composition of human faeces are so unending that these experiments cannot be held to dispose of this difficulty, and it is indeed highly probable that this factor does contribute to discrepancies between results obtained in different laboratories.

It should also be observed that the spacing of dilutions in the Chick-Martin test places limits on its accuracy which are wider than is perhaps fully understood. However perfectly devised and executed a test may be, there must be one phenol dilution and one dilution of the disinfectant tested in which bacteria may or may not survive. In a Chick-Martin test, for example, a positive culture may or may not be obtained from the tube containing 0·92 c.c. 5 per cent. phenol (in a total volume of 5 c.c.) and the disinfectant tested might or might not give a positive culture from the tube containing for instance 1·08 c.c. of a 2 per cent. solution. There are four possible combinations of these alternatives, and the resulting coefficients are 2·12, 2·52, 2·53 and 3·00.

The average of these is 2·54; the lowest represents a difference of −16·5 per cent. and the highest of +18·1 per cent. Slightly different results are obtained from similar calculations to this at other points in the range of possible dilutions, but it is correct to say that the Chick-Martin test cannot by definition, and apart altogether from any sources of actual error, be worked to any narrower limits of accuracy than approximately ± 17 per cent. These limits are undesirably wide.

There are therefore good reasons for attempting to devise a method of testing disinfectants which shall serve the same purpose as the Chick-Martin test while eliminating its defects.

A PROPOSED METHOD EMPLOYING YEAST AS ORGANIC MATTER

In the light of the foregoing observations it would probably be feasible to improve the reliability of the Chick-Martin test, if only by complete restoration of the faecal suspension to its original state of subdivision. There is however no certainty that the desired degree of reliability would thus be achieved, since the composition of the organic matter used remains a variable factor; the aesthetic and hygienic objections still apply, and the time expenditure remains excessive. If organic matter were available in an equivalent form to which these objections do not apply, it should be used in preference to faeces. The argument leading to the proposal that this alternative form of organic matter should be a suspension of commercial yeast has already been stated (Garrod, 1934), and some evidence has been given that this material will serve the purpose. The reduction of the activity of disinfectants by a suspension of faeces is due mainly to the small particles in this suspension. These particles consist chiefly of bacteria (which constitute a substantial proportion of the total dried weight of faeces); a suspension of bacteria only should therefore have the same effect. A sufficient amount of bacteria for the purpose of such a test could only be obtained from impracticably large quantities of culture, but an equivalent material is everywhere obtainable cheaply and in ample quantity in the form of commercial yeast. The examination of ten samples from different sources by a variety of methods has shown that the composition of yeast is constant within narrow limits, and it has the supreme advantage (in view of previous observations on the behaviour of faecal suspensions and its results) of almost constant and entirely unalterable particle size.

The chemical composition of yeast. No attempt appears to have been made to define exactly the degree of complexity in chemical composition which must be required of "organic matter" for use in a test for disinfectants. No material of constant composition could conceivably emulate in complexity the hotch-potch of varied food residues, digestive secretions, bacteria, and products of decomposition which constitute faeces; yet it is not known that this variety of material is really necessary in order to determine the extent to which disinfectant activity will be reduced in the presence of faeces *per se*; many of its constituents have probably little effect in this direction. It is generally

considered that the organic matter used should be present in both a particulate and a dissolved condition, and that it should be of such a nature as to adsorb or combine with the active elements in disinfectant solutions, reducing their germicidal activity to an extent equivalent to that occasioned in severe but reasonable conditions of practical use. An autoclaved yeast suspension containing 20 g. moist yeast per 100 c.c. contains about 1·4 per cent. of solids in solution, the remainder of the material being in particulate form, and reduces the activity of a coal-tar disinfectant to a slightly greater extent than the faecal suspension used in the Chick-Martin test.

That the chemical composition of yeast is sufficiently complex and representative for the purposes of a disinfectant test may perhaps be deduced from the following statement, for which I am indebted to Dr J. Vargas Eyre, Ph.D., F.I.C., of the Research Department of the Distillers Company Limited.

"The approximate composition of the dry matter of a good yeast made by modern methods is as follows:

			%
Nitrogenous bodies (such as protein, nucleic acid, etc.) ...	...	...	52
Carbohydrates ("Hemicellulose", glycogen, mannan, etc.)	...	...	31
Fat	...	...	7
Ash	...	...	10

A typical ash would have the following composition:

	%		%
K_2O	3·62	MgO	0·44
CaO	0·10	P_2O_5	4·21

with traces of silicates, sulphates, lead, zinc, iron and copper."

The preparation of yeast suspensions. In preliminary experiments previously reported the suspension used contained 20 g. of moist yeast per 100 c.c. Since there is a slight variation in the water content of yeasts bought from retail sources, it is preferable to standardise the suspension by dry weight, and in subsequent experiments the concentration of the suspension used has been equivalent to 5 g. dry weight per 100 c.c. Since this is somewhat weaker than a 20 per cent. moist weight suspension, the latter can be prepared first; the ratio of dry to moist weight is then determined by drying a small sample to constant weight at 55° C., and the necessary adjustment of the strength of the suspension can then be made by adding the required amount of distilled water.

Example. 100 g. fresh moist yeast was made up to 500 c.c. with distilled water and autoclaved. 2 g. of the yeast was dried to constant weight, which was found to be 0·555 g. The concentration of the suspension made was therefore 5·55 per cent. by dry weight and the addition of 55 c.c. distilled water was necessary to bring it to a 5 g. per cent. dry weight content. This method obviates the disadvantages of drying the whole of the yeast used.

An alternative method is to use dried yeast commercially prepared, in which case the material is simply suspended in distilled water in the proportion of 5 g. per 100 c.c. This simple method has two drawbacks. Dried yeast is not a product in common use, and in the event of its being employed in a generally

adopted and standard test would possibly have to be obtained from a specified source in order to ensure constancy in composition. Its second disadvantage is that it does not readily form a good suspension, and although after autoclaving visible masses are found to have disintegrated, microscopic examination shows that clumps of some scores or hundreds of cells are distributed singly. Further disintegration can only be secured by prolonged shaking. No evidence, however, has been obtained that the existence of these microscopic clumps alters the capacity of the suspension to reduce disinfectant activity. This question will be dealt with further in a subsequent section. By whichever of these methods a suspension is obtained, it is autoclaved at 15 lb. for 30 min., and subsequently neutralised. The pH after autoclaving varies from 6·1 to 6·4, and not more than 2 c.c. $N/1$ NaOH pr 100 c.c. are usually required in order to bring it to 7·0, the reaction which was chosen for the tests to be described. This addition, and subsequent distribution in small flasks, can be carried out without danger of contamination, but the flasks can be re-sterilised by a short period of steaming if this is considered necessary. Sterile yeast suspensions appear to retain their properties unchanged in cold storage; no difference in action has been detected between freshly made suspensions and others up to three months old, provided that evaporation has been prevented.

The addition of culture to yeast suspension. In the Chick-Martin test organic matter and disinfectant dilutions are first mixed, and culture is added to this mixture after an unspecified interval of time at the commencement of the test proper. This is theoretically an unsound proceeding, since in practical disinfection there is no such separation of bacteria and organic matter, the process of disinfection and the process of inactivation of the disinfectant by organic matter proceed simultaneously. It is not certainly known whether the period of contact between disinfectant and faecal suspension before the Chick-Martin test begins can influence the result, but such an influence is theoretically possible. In order to obviate this possible source of error and at the same time to bring the proposed test in line with practical conditions, the proceeding adopted has been the previous admixture of culture and organic matter. In the tests described this mixture was made by adding 1 part of a 24-hour culture of *B. typhosus* ("S" strain) in Rideal-Walker broth to 24 parts of yeast suspension in a small flask. When 2·5 c.c. of this mixture are added to 2·5 c.c. of diluted disinfectant, the amount of culture in the total volume of 5 c.c. remains 0·1 c.c., as in the Chick-Martin test, but the separate measurement of this small volume for each tube used in the test is obviated. Before this procedure can be accepted as satisfactory, it must be known that typhoid bacilli in a yeast suspension kept at 20° C. neither die nor multiply unduly within such a period as is likely to elapse before the mixture is actually used. The following experiment settles this question:

To 24 c.c. of an autoclaved and neutralised 5 g. dry weight per cent. yeast suspension was added 1 c.c. of a 24-hour culture of *B. typhosus* "S". The small flask containing this mixture was kept in a water-bath at 20° C. and at intervals

1 c.c. was diluted with 99 c.c. of sterile distilled water, and 1 c.c. of this dilution with a further 99 c.c.; of this second 1 in 100 dilution 0·5 c.c. was used as inoculum for a shake agar culture, which thus represented 1/20,000 c.c. of the original material. The following colony counts in these cultures resulted:

On addition of culture to yeast suspension					288
After 30 min.	...	...	...	...	263
„ 90 „	...	...	...	...	315
„ 3 hours	...	...	...	...	378
„ 5 „	...	...	...	...	511
„ 24 „	...	...	...	...	+ + (about 20,000)

The initial fall in these counts is within the limits of experimental error; subsequent counts show that yeast suspension is a medium in which typhoid bacilli multiply, but so slowly at a temperature of 20° C. that no material change in numbers is to be expected if the mixture is made within any reasonable period of time before the test itself is begun.

The preparation of disinfectant dilutions. Various systems of dilution have been proposed for disinfectant tests. In these there are anomalies which are inevitable with the type of method employed, and with any system based on round numbers. In the Rideal-Walker series of dilutions, for example, the volume proceeds by increments of 50 from 1 in 100 to 1 in 500, and by increments of 100 thereafter. The degree of difference in effective concentration between the successive dilutions of 1 in 100 and 1 in 150, or 1 in 400 and 1 in 500, and 1 in 2400 and 1 in 2500, is obviously quite disproportionate; these three examples represent increases in dilution of 50 per cent., 25 per cent. and 4·16 per cent. respectively. The system employed in the United States Hygienic Laboratory test (Report of U.S. Hygienic Laboratory, 1921) is more elaborate, but open to the same objection in a lesser degree; the volume increment from dilutions of 1 in 70 to 1 in 160 is 10, thence to 1 in 200, 20, thence to 1 in 400, 25, thence to 1 in 900, 50, thence to 1 in 1800, 100, thence to 1 in 3200, 200. Of an almost identical system which preceded this it was said (Park, 1918) that the "increment is 10 per cent. of the number occupying the position of geometrical mean of each group, and 7 per cent. and 12·5 per cent. respectively of the end numbers of the group". Neither this irregularity in progress nor the elaborate proceedings entailed in preparing such dilutions is necessary.

It is clearly desirable that there should be a constant difference between any two consecutive dilutions in such a series. This difference can only be constant if the quantity of disinfectant in a given volume of each dilution is reduced at each step in the series by the same fraction of the quantity in the previous dilution. The only convenient method of preparing such a series, and one which is simplicity itself, is to remove a constant volume from the original solution and replace it with the same volume of distilled water, repeating this operation until the whole series of dilutions desired has been obtained. Any series of dilutions can then be prepared in a single stoppered measuring cylinder, by the use of only two pipettes, with the greatest accuracy, since only comparatively large volumes are being measured, and without any possibility of a mistake being made.

The method employed in these experiments was the initial preparation of a 2 per cent. dilution of the disinfectant to be tested by making up 5 c.c. (measured by means of an accurate capacity pipette, which is thoroughly washed out in the distilled water used for dilution) to 250 c.c. with distilled water in a graduated stoppered cylinder. After thorough mixing a further dilution in bulk is made in a second cylinder to furnish a solution of the first strength required for the test, the volume of this second dilution being adjusted to 100 c.c. (*e.g.* if the first dilution to be used in the test were 1·18 per cent., 59 c.c. would be made up to 100 c.c. with distilled water). Of this solution 10 c.c. are removed by means of a graduated 10 c.c. pipette, and 2·5 c.c. are placed in either one or more tubes to be used in the actual test, the remainder being discarded. With a separate 10 c.c. pipette, 10 c.c. of distilled water are added to the cylinder, restoring the volume of disinfectant solution in it to 100 c.c. After thorough mixing (by repeated inversion of the cylinder) a further 10 c.c. are removed and replaced with distilled water, this process being continued until the whole range of dilutions required has been obtained. Phenol dilutions are prepared in the same way, the initial bulk dilution being prepared from a stock 5 per cent. solution.

For a range-finding experiment wider spacing of dilutions may be employed, and obtained for instance by removing and replacing 25 per cent. of the total volume at each step in dilution. For the test itself the requisite degree of accuracy demands a reduction of not more than 10 per cent. at each step, a degree of difference between successive dilutions similar to that specified for the Rideal-Walker and United States Hygienic Laboratory tests, and considerably less than that employed in the Chick-Martin test. Whether any advantage is to be gained from still closer spacing has not been determined. The following are the concentrations resulting from the successive dilution by this method of an initial 2 per cent. solution, the series being carried to approximately 0·2 per cent., a point beyond which it is not likely to be necessary to dilute any known disinfectant for the purpose of this test:

Initial dilution	2 %	= 1 in 50
Dilution 2	1·8	55·5
3	1·62	61·7
4	1·458	67·6
5	1·312	76·2
6	1·180	84·7
7	1·062	94·1
8	0·956	104·6
9	0·860	116·3
10	0·774	129·2
11	0·697	143·4
12	0·627	159·5
13	0·564	177·3
14	0·508	196·8
15	0·457	218·8
16	0·411	243·3
17	0·370	270·2
18	0·333	300·3
19	0·300	333·3
20	0·270	370·3
21	0·243	411·1
22	0·218	458·6
23	0·196	507·7

15–2

Were this system of dilution adopted for any test requiring the use of higher dilutions of disinfectant, a fresh series could be begun with a 0·2 per cent. solution, which would then correspond with the series given, the figures being the same after one shift of the decimal point.

The sacrifice of round numbers which the use of this system involves is of no consequence, especially since these figures do not appear in the stated result of a test, and therefore need not convey an impression of pedantic (and
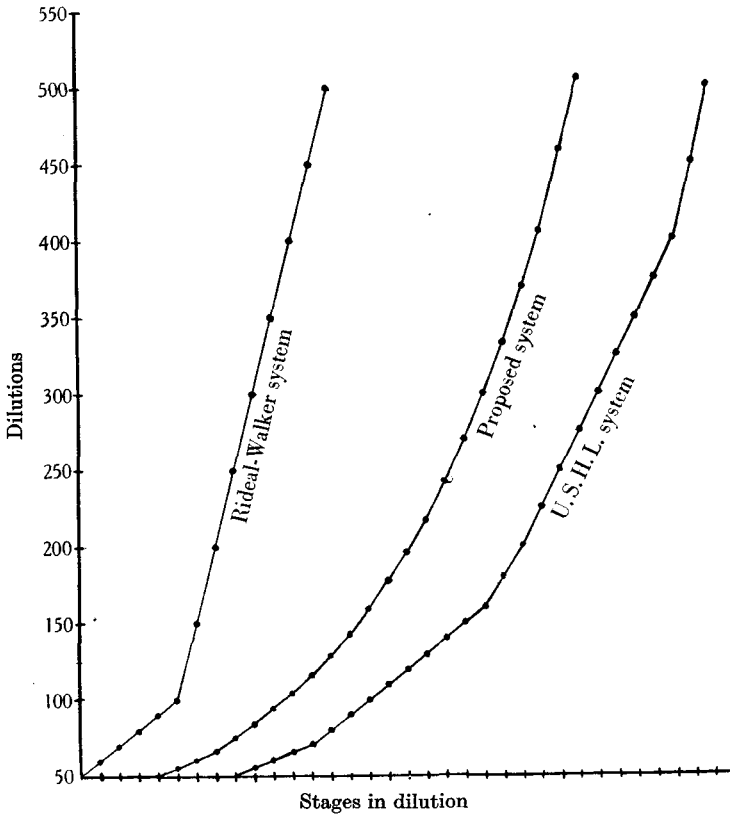

Fig. 1. Graph showing series of dilutions prepared by three different methods.

impossible) accuracy to the uninstructed. The result of the test is a coefficient, obtained by dividing the highest dilution of disinfectant from which a negative culture is obtained by the corresponding dilution of phenol. Thus if these dilutions were respectively 1 in 270·2 and 1 in 67·6, the resulting coefficient expressed to the nearest first decimal point is 4·0.

In Fig. 1 the stages in this series of dilutions are plotted as a graph, together with the dilutions prescribed within the same range for the Rideal-Walker and United States Hygienic Laboratory tests. The graph of the proposed system forms an exponential curve, whereas those of the other two systems proceed

in a series of straight lines with abrupt transitions between them. It may reasonably be claimed that this method of making dilutions is not only mathematically sound, but simpler in execution than other methods in common use, and its employment for other purposes than that of this particular test might be found advantageous.

The performance of the test. The test itself has consisted of adding the mixture of yeast suspension and culture to equal volumes of disinfectant dilutions and phenol dilutions, and cultivating these mixtures after 30 min., during which period they are kept at a temperature of 20° C. The disinfectant and phenol dilutions, of each of which 2·5 c.c. have been prepared as already described, have been contained in plugged test-tubes, but it is intended to experiment with the use of rimless tubes with glass caps, which would obviate the flaming necessitated by the use of cotton-wool plugs. For the addition of the mixture of yeast suspension and culture to these tubes a new method has been employed. Such additions in other tests are invariably made by means of a pipette, but the comparatively large volume concerned here makes it possible to use a burette, and this has been found convenient and more easily worked. The mixture of 24 parts of yeast suspension with 1 part of culture (convenient actual quantities for a test would be 48 c.c. and 2 c.c. respectively) is kept in a small flask in the water-bath at 20° C. and run into a burette through a funnel immediately before the test is to begin. The tubes containing disinfectant dilutions are also placed in the bath beforehand for a sufficient time for them to attain the correct temperature. A volume of 2·5 c.c. of fluid from the burette is then run into each tube at intervals of 30 sec. It is advisable that the tube be held vertically beneath the burette in order that the added mixture may flow directly into the disinfectant and not down the side of the tube; even so the tube should subsequently be "rolled" at an angle of about 30° in order to ensure that none of the inoculum is left on the side of the tube beyond the reach of the disinfectant. After sufficient gentle shaking to ensure even admixture the tube is then returned to the water-bath. The use of a burette or other device for delivering measured volumes of mixed yeast suspension and culture from a considerable bulk may be criticised on the ground that deposition may occur in the mixture. Actually the formation of deposit in a yeast suspension is so slow a process that only quite undue delay could enable this factor to come into operation.

After a period of 30 min. in the case of each tube one 3 mm. loopful of its contents is removed and sown in a tube of Rideal-Walker broth; possible modifications of the loop used and of this culture medium are discussed in succeeding paragraphs. The cultures so made are incubated at 37° C. for 48 hours, and the results having been read, the coefficient is calculated by dividing the highest dilution of disinfectant which produces sterility by the highest dilution of phenol producing sterility. Thus if, as in an example already given, these dilutions are respectively 1 in 270·2 and 1 in 67·6, the coefficient is 720·2/67·6 or approximately 4·0.

The loop. It is necessary for well-known reasons to cultivate only a very small volume of the test mixture, and such a volume cannot conveniently be measured by a pipette. The wire loop is a convenient instrument for this purpose in many ways, but it is by no means an instrument of precision. Although detailed studies of its performance have been made, both these and the improvements suggested in consequence have been inexplicably ignored in this country. Duyser and Lewis (1914) found that the volume of fluid transferred by a 4 mm. loop, as employed in the Rideal-Walker test, varied from the mean commonly by 30 per cent. and sometimes as much as 80 per cent. St John (1914), in view of these findings, suggested using a wire cube with sides 3 mm. in length; the error with this instrument was only ± 6 per cent. A greater improvement is represented in the loop used for the United States Hygienic Laboratory test; this consists of four contiguous turns of nichrome wire of No. 23 B and S gauge (0·0226 in. in diameter) wound as tightly as possible round a wire of No. 13 B and S gauge (0·072 in. in diameter). The limits of variation with this loop are stated as $\pm 2·71$ per cent. (Park *et al.*, 1918). Since this loop can claim a higher degree of demonstrated accuracy than any other, and since there is advantage in any step towards international uniformity of practice, the adoption of this loop for any standard disinfectant test to which its use is applicable is worthy of consideration.

The culture medium. It is essential that the broth used for a disinfectant test should be of standard composition attainable in any laboratory. There is abundant evidence (*e.g.* Rideal and Walker, 1903, 1913; Wright, 1917; Süpfle, 1918; Park *et al.*, 1918; Report of Royal Institute of Public Health, 1919) that the resistance of bacteria to disinfectant action depends very largely on the medium in which they are grown, and within surprisingly wide limits. Even comparatively slight changes in composition can have effects disastrous to the reliability of a disinfection test. The perfect solution to this difficulty would be a purely synthetic medium, but apart from the fact that such media considerably increase the susceptibility of members of the *Bacterium* group to disinfectant action,[1] they have the still greater disadvantage of including ingredients which, if obtained in a state of sufficient purity, are quite prohibitive in cost. Among media containing peptone and meat extract, there is only one in this country which can be relied on as constant in composition, the manufacturers of these two ingredients having undertaken to maintain them as standard and unvarying products. This medium is Rideal-Walker broth. It has been criticised in the past as a poor medium, in the sense that growth in it may be slow and comparatively scanty. Actually a normal 24-hour culture of *B. typhosus* in this medium is quite obviously turbid, but the amount of growth is admittedly less than may be obtained in other broths. Wright (1917) has suggested an explanation for this. He points out that the sodium chloride content is excessive; 1 per cent. is added, and the meat extract (Lab. Lemco) itself contains 2·6 per cent., which will further slightly increase the salt

[1] Personal observation.

content of the medium. He is also of the opinion that the quantities of meat extract (2 per cent.) and of peptone (2 per cent.) are excessive. (The broth used in the United States Hygienic Laboratory test contains 0·3 per cent. of Liebig's meat extract, 1 per cent. of Witte's peptone, and 0·5 per cent. of added sodium chloride.) In order to test the validity of these criticisms, the following experiment was carried out:

Four broths were prepared, and equal volumes of each were inoculated with one 2 mm. loopful of a broth culture of *B. typhosus*. After 24 hours' incubation, three serial 1 in 100 dilutions in sterile distilled water in flasks were made from each of these cultures and duplicate shake cultures in agar from 1 c.c. and 0·2 c.c. of the last (these cultures thus containing 1/1,000,000 c.c. and 1/5,000,000 c.c. of the original cultures). Colonies were counted after 48 hours' incubation and the results averaged. The living bacterial content of each of these four broths was found to be as follows:

Broth containing 1 per cent. Witte's peptone, fresh beef heart extract and 0·5 per cent. sodium chloride (as used for routine purposes in this laboratory).

884 millions per c.c.

Rideal-Walker broth (2 per cent. Eupepton, 2 per cent. Lab. Lemco and 1 per cent. sodium chloride).

376 millions per c.c.

Rideal-Walker broth modified as follows: 2 per cent. Eupepton, 2 per cent. Lab. Lemco and 0·5 per cent. sodium chloride.

579 millions per c.c.

Rideal-Walker broth modified as follows: 1 per cent. Eupepton, 0·5 per cent. Lab. Lemco and 0·5 per cent. sodium chloride.

538 millions per c.c.

As far as they go these observations clearly confirm Wright's criticisms that the sodium chloride content of Rideal-Walker broth is excessive; it is indeed difficult to understand why a concentration as great as 1 per cent. was ever proposed. Of the possible advantage of reducing the amount of peptone and meat extract there is no evidence, but this is a matter which merits further investigation.

It is therefore suggested that Rideal-Walker broth be employed in this test, for the sole reason that it is the only available medium the composition of which should be unvarying wherever it is made; that the concentration of added sodium chloride be reduced to 0·5 per cent. and that the concentrations of peptone and meat extract may subsequently also be reduced in the interests of both economy and efficiency should further study show that such changes are advisable.

The optimum concentration of yeast suspension. In the method described the concentration of the yeast suspension suggested is 5 g. of dried yeast per 100 c.c. This concentration was originally employed because it corresponds roughly to the quantity of organic matter used in the Chick-Martin test, both in actual dried weight per unit of volume and in its effect in diminishing the

activity of disinfectants; to be precise, the amount of yeast is 1 per cent. less and its capacity to diminish disinfectant action is somewhat greater than that of the dried faeces. It may be asked whether a lesser concentration of organic matter might furnish results of greater value, since the range of coefficients obtained would be wider, and a distinction could therefore more readily be drawn between the merits of one disinfectant and another. Before this question can be decided it is necessary to know how the coefficient obtained varies with different concentrations of yeast suspension. The following experiment was carried out to ascertain this:

The experiment was performed with three different disinfectants in addition to phenol, and nine different concentrations of yeast suspension. The disinfectants which will subsequently be referred to as Fluids A, B and C were

Fluid A. A lysol.

Fluid B. A black fluid with a R.-W. coefficient of 6–7.

Fluid C. A black fluid with a R.-W. coefficient of 18–20.

The yeast suspensions were prepared from an original 5 per cent. suspension by removing one-quarter of its volume and replacing it with sterile distilled water; this operation was repeated eight times, thus furnishing a series of nine equally graded dilutions ranging from 5 per cent. to 0·5 per cent. A tenth experiment was performed in which the yeast suspension in the mixture with culture was replaced by distilled water. Tests otherwise exactly as described were then performed with all three disinfectants with phenol controls, using each of these ten suspensions. The results obtained can best be briefly represented by tabulating the phenol coefficients which they indicate.

Phenol coefficients obtained with varying concentrations of
yeast suspension

Disin-fectant	Concentration of yeast suspension in g. of dried yeast per 100 c.c.									
	5	3·75	2·81	2·10	1·57	1·17	0·88	0·66	0·5	Nil
Fluid A	1·54	1·72	1·91	1·91	1·91	2·12	2·12	2·12	2·12	2·12
B	2·62	2·91	3·23	3·59	3·99	3·99	4·44	4·44	4·44	4·86
C	4·44	4·93	6·08	6·78	7·40	8·23	8·23	9·11	9·11	12·61

These results clearly illustrate facts which are already familiar, namely that the action of dissolved coal-tar disinfectants is much less affected by the presence of complex organic matter than that of emulsified coal-tar disinfectants, and that, among the latter, the higher the efficiency as indicated by a Rideal-Walker coefficient the greater is the proportional reduction in efficiency when organic matter is introduced. The disinfecting capacity of these three fluids bears the same mutual relationship whatever the quantity of organic matter, but the *magnitude* of the indicated difference varies with this quantity; at the maximum concentration of yeast suspension (5 per cent.) the ratio between the three coefficients is as 10 : 14 : 28; at the least concentration (0·5 per cent.) it is as 10 : 21 : 43.

The choice of an optimum concentration is really a matter of sanitary policy. How often are disinfectants used in the presence of such quantities of

organic matter as a 5 g. per cent. suspension of yeast represents? If these or even more unfavourable conditions are of frequent occurrence, it is clearly advisable to make a test as severe as possible, and an even greater concentration of organic matter than 5 per cent. might be considered necessary. If on the other hand common types of use represent more favourable conditions, it is better to credit fluids having a high Rideal-Walker coefficient with the higher efficiency which they display in the presence of smaller quantities of organic matter. While this question must be a matter of opinion, there are strong arguments in favour of a test of considerable severity, and the 5 per cent. yeast suspension used may be held to meet this demand.

EXPERIMENTS TO DETERMINE THE RELIABILITY OF THE METHOD

A method such as this can only finally be accepted when it has stood the trial of extended experience. It may however be submitted to forms of examination which will detect specific defects if they exist. In view of demonstrated consequences in the Chick-Martin test of the formation of a deposit in the test mixture, it is necessary to know whether the same considerations apply to a mixture containing yeast. As a general test of reliability it is of some use to carry out multiple identical tests and compare their results. In these two directions the following data have been obtained.

Deposition in the test mixture. In a mixture of 5 per cent. yeast suspension and disinfectant slow deposition of yeast cells takes place, and at the end of 30 min. a creamy deposit is formed representing a fraction (by no means the whole) of the yeast in the mixture. In order to determine whether this deposit exerts an undue protective action on bacteria contained in it, an experiment was performed identical with that in which this action was demonstrated in the case of a suspension of dried faeces:

To four tubes each containing 2·5 c.c. of a 5 per cent. yeast suspension was added 2·5 c.c. of each of four dilutions of a disinfectant (the "white fluid" used in experiments previously described). 0·1 c.c. of a 24-hour broth culture of *B. typhosus* "S" was then added to each tube, and they were maintained undisturbed at a temperature of 20° C. for 30 min. Separate dilutions and shake cultures were then made from both the supernatant fluid and the deposit in each tube, care being taken not to disturb the deposit until the supernatant fluid to be used for culture had been removed. The colony counts in these cultures gave the following results:

Numbers of typhoid bacilli per c.c. surviving in mixtures of yeast suspension and disinfectant

Quantity of 2 % disinfectant in mixture c.c.	In supernatant fluid	In deposit
0·55	2,820,000	2,760,000
0·66	930,000	1,080,000
0·77	88,000	92,000
0·92	400	200

Control (0·1 c.c. culture in 5 c.c. water): 4,620,000

The differences between the counts of surviving bacteria in the two portions of the mixture in this experiment are within the limits of experimental error, and it may be concluded that the progress of disinfection is uniform throughout the contents of the tube. This is no doubt due to the fact that the deposit in a tube containing yeast suspension is small, fluid and slowly formed, whereas in a mixture containing dried faeces it comprises almost the whole of the solid content of the mixture, and forms a compact mass within a few seconds, thus immediately removing a small proportion of the bacilli from free contact with the disinfectant. It may be concluded from this experiment that the formation of a deposit in mixtures containing yeast suspension is not a source of potential error, and shaking is unnecessary except to secure even admixture at the outset.

The extent of variation in the results of multiple tests. It has already been pointed out that any test depending on complete sterilisation must give results inevitably varying within certain self-imposed limits. These limits, which depend on the spacing of the dilutions used, can readily be calculated; for the Chick-Martin test they are approximately ± 17 per cent. The same calculation applied to the proposed test shows that its corresponding limits of possible variation are approximately ± 10 per cent.

Examples. If the phenol tube, in which the number of surviving bacilli may be so small that either a positive or a negative culture may result, be taken as that containing 1·31 per cent. phenol, and the corresponding tube in the disinfectant range that containing 0·50 per cent., then the four possible coefficients are 2·33, 2·58, 2·62 and 2·91; the average of these is 2·61 and the extremes are +11·1 per cent. and −10·7 per cent. In the case of a fluid yielding a higher coefficient, the possible limits are slightly wider; taking the doubtful phenol dilution again as 1·31 per cent. and the corresponding disinfectant dilution as 0·33 per cent. the four possible coefficients are 3·54, 3·94, 4·0 and 4·44; average 3·98, extremes +11·55 per cent. and −11·05 per cent. These are therefore the limits within which variation in the resulting coefficient is possible apart from the influence of any source of actual error.

Several series of multiple tests have been carried out in order to determine what amount of variation is to be expected in practice. These experiments were of two kinds; those in which the same dilutions and the same yeast suspension were used for a series of successive tests, in which therefore variation should only be the result of minor inaccuracies in manipulation or chance as represented by the tube containing few surviving bacilli, and a series of tests in which a different yeast suspension was used for each, which should therefore indicate how far the possible inconstancy of different samples of yeast can affect the coefficient obtained.

Ten sets of appropriate dilutions of phenol and of Fluid B were prepared, and ten flasks of the mixture of culture and yeast suspension, the same culture and the same yeast suspension being used for all. Ten successive tests were then carried out. The coefficients obtained were 2·36, 2·58, 2·58, 2·62, 2·62, 2·62, 2·62, 2·62, 2·91, 2·91. The average of these is 2·64, the average variation is ± 3·9 per

cent. and the extreme variations from the mean are $+10.2$ per cent. and -10.5 per cent. The same proceeding was then carried out using ten different yeast suspensions. Two of these had been made at different times from the same sample of dried yeast, and eight from different samples, largely from different sources, of moist yeast. The same disinfectant dilutions and culture were used throughout. The coefficients obtained were 2·33, 2·36, 2·36, 2·58, 2·58, 2·58, 2·87, 2·87, 2·91, 2·91. The average of these is 2·63, the average variation is ± 7.6 per cent. and the extreme variations are $+10.6$ per cent. and -11.4 per cent. Identical experiments with Fluid C gave the following results: using the same yeast suspension throughout, the coefficients given by ten tests were 3·59, 4·00, 4·00, 4·00, 4·00, 4·43, 4·43, 4·44, 4·44, 4·44; average coefficient is 4·17, average variation ± 6.0 per cent., extreme variations $+6.4$ per cent. and -13.8 per cent. Using ten different yeast suspensions the coefficients were 3·59, 3·59, 3·94, 3·94, 3·94, 4·00, 4·00, 4·00, 4·43, 4·43; average coefficient 3·98, average variation ± 4.5 per cent., extreme variations $+11.3$ per cent. and -9.8 per cent.

The degree of variation in the results of multiple tests was therefore within the limits imposed by the spacing of the dilutions, although in fact the occurrence sometimes of positive and sometimes of negative cultures was not confined to one dilution of the disinfectant, but involved two, and in the case of one yeast suspension (which may have been concentrated somewhat by evaporation during storage) a third. The average variation from the mean is small, and although it is somewhat more in the case of Fluid B when different yeast suspensions are used, there is not a corresponding difference in the results with Fluid C. As evidence that this method is reliable it should be added that "irregular tubes" very rarely occur.

These results therefore indicate that the method is workable and yields at least reasonably consistent results. Further and more exact definition of procedure should be capable of ensuring a degree of accuracy greater than these preliminary findings exhibit.

The behaviour of different types of yeast suspension. In some recent experiments attempts have been made to determine whether there is any distinguishable difference between the action of suspensions made from dried and from moist yeast, and in the case of the former, between that of suspensions containing clumps of cells and those in which these clumps have been broken down. The method employed has been the enumeration of surviving bacilli at different time intervals in disinfectant dilutions such that complete sterilisation occupies a considerable period of time; this method served previously to illustrate clearly the effect of varying the physical state of the faecal suspension used in the Chick-Martin test. These experiments need not be described, since they failed to indicate any significant difference between the action of yeast suspensions prepared in different ways. The explanation of this uniformity in action is presumably the constant size of the particle in the suspension, and the narrow limits within which the number of these particles varies.

DISCUSSION

These findings are presented not as final conclusions, but as forming a basis upon which an acceptable method for testing disinfectants may be founded. The essential feature of the method, the employment of yeast instead of faeces as organic matter, has not been found to possess any unexpected drawbacks, and has important advantages. The chief of these is that yeast exhibits a degree of regularity in composition and action unusual in a natural product, and unapproached by any material hitherto employed which has an equivalent effect.

CONCLUSIONS

1. Methods of testing disinfectants which introduce faeces as added organic matter are subject to error.

2. A method in which a suspension of yeast is used for this purpose causes an equivalent reduction in disinfectant activity, and yields consistent results.

3. Other departures from existing practice are proposed with a view to simplifying the procedure of such a test and improving its accuracy.

This report is a contribution to the work of Committee C/10/1/1 of the British Standards Institution, the terms of reference of which are to investigate the feasibility of the proposal, made originally by the writer, that yeast should be substituted for faeces in a disinfectant test. The parent Committee C/10/1 exists to prepare a technique for testing disinfectants in the presence of organic matter which shall be adopted as a British Standard Method. The disinfectants used in the experiments described were supplied to all members of Committee C/10/1/1 by Mr G. A. Freak, B.Sc., F.I.C., of the Cooper Technical Bureau, who is a member of this Committee. The dried yeast employed was kindly supplied by Dr J. Vargas Eyre, Ph.D., F.I.C., of the Research Department of the Distillers Company Limited.

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