THE EFFECT OF THE SHAPE OF CONTAINER AND SIZE OF GAS TUBE IN THE PRESUMPTIVE COLIFORM TEST

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CONTENTS

Part I. Description of experiments

'Acid and Gas' are the accepted evidence of a positive result in the presumptive coliform test, and the normal practice is to entrap the gas in a Durham tube. It would seem possible that variations in the size of gas tube and shape of container might affect the number of positive results, but although general use is made of tables such as McCrady's, which give the most probable number of coliform organisms, and although there is an increasing tendency to demand greater 'exactitude' in the coliform test by insisting that reports be based on such tables, no work seems to have been done on the fundamental problem of finding how far the number of positives is affected by changes in the container and gas tube, or indeed of determining whether, in actual practice, a positive result follows a single cell inoculation, or whether a greater number of cells is required to give a positive result.

Correspondence and a search of literature have revealed only two references on the subject. Morgan & Holmes (1927) reported observing considerable variation in the coliform content of the same milk when different methods of collecting gas were used. The saccharimeter and J tube collected most gas, then the long type of Durham tube and test-tube. The least gas was collected in the $\frac{1}{2}$ in. test-tube and small Durham tube. They stated that the latter method might show absence of coliform organisms when possibly the other three methods showed presence in 1 ml. or $\frac{1}{10}$ ml. Chalmers (1928) isolated coliform organisms from plates of 1/10 dilution of milk samples which had given a negative presumptive coliform test in two out of three tubes at $\frac{1}{10}$ ml. and thought that part at least of the inaccuracy of the coliform test might be due to the structure of the ordinary Durham tube, which failed to ensure

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the collection of the gas produced. Barkworth & Irwin (1938) investigated the accuracy of the coliform test. Their results, in which each of three workers made seventeen tests on each of seven samples, each test consisting of five-fold inoculation at four different levels, showed that a wide variation might occur in the proportion of positive and negative tubes, though not at any one dilution in excess of that expected by chance.[1]

Apart from these observations, we can trace no systematic work on the effect of size of gas tube on the number of positive results.

There is also no general agreement as to size of gas tube. The Ministry of Health (1937) and (1939) recommended a $6 \times \frac{5}{8}$ in. test-tube and a $1\frac{3}{8} \times \frac{5}{16}$ in. gas tube, using 5 ml. of medium and 1 ml. of inoculation, and the same size of gas tube is suggested for a $6 \times \frac{3}{4}$ in. test-tube (1939) using 10 ml. of broth and 10 ml. of inoculation. With inoculation of 50 ml. of water (1939) a $3 \times \frac{7}{16}$ in.[2] gas tube is advised and a 6 oz. medicine bottle. With this last-named combination the gas tube usually rests diagonally across the bottle at an angle of about 55°. The Ministry of Agriculture (1934) recommend a $2 \times \frac{3}{8}$ in. rimmed gas tube and a $6 \times \frac{5}{8}$ in. test-tube, using 6 ml. of medium and 1 ml. of inoculation, and Mackenzie (1940) reports the use of a $2 \times \frac{1}{2}$ in. gas tube with a $6 \times \frac{3}{4}$ in. test-tube.

When testing 50 ml. of water it is possible to use other containers, such as a 1 or $1\frac{1}{2}$ in. test-tube, or a bottle of cylindrical shape, such as an 8 oz. kali bottle which has a diameter of about 2 in.

The diversity of recommendations as to suitable combinations of container and gas tube lead to speculation as to the determining factors. Is it the 'fit' of the gas tube against the bottom of the test-tube that matters, or the ratio of the diameter of the test-tube to the diameter of the gas tube, or the ratio of the total volume of liquid medium plus inoculum to the volume of the liquid within the gas tube? The ratio of the length of the gas tube to the depth of the medium in the container depends on the last two factors, if the tube is not completely immersed.[3]

Let us consider the combination of $6 \times \frac{5}{8}$ in. test-tube and $1\frac{3}{8} \times \frac{5}{16}$ in. gas tube. The capacity of the gas tube is 1·3 ml. or 22 % of the 6 ml. of liquid used. This ratio is approximately preserved with a $1\frac{1}{2}$ in. test-tube and 100 ml. of liquid if we use a $4 \times \frac{3}{4}$ in. gas tube. The latter has a capacity of 21 ml.

The greatest opportunities for varying the size of both gas tube and container occur when testing quantities of 50 ml. which in practice are inoculated into 50 ml. of (double-strength) broth, thus giving a total of 100 ml.

[1] $6 \times \frac{5}{8}$ in. test-tube and gas tube $2 \times \frac{3}{8}$ in. (rimmed).

[2] These are usually rimless.

[3] If the total volume of the liquid is V ml. and d_c, d_g and l_g cm. are respectively the diameter of the container, the diameter of the gas tube and the length of the gas tube, then, neglecting the volume of the glass, the depth of the medium in the container is approximately $D = \frac{V}{\frac{1}{4}\pi d_c^2}$ if the tube is completely immersed and $D = \frac{V - \frac{1}{4}\pi d_g^2 l_g}{\frac{1}{4}\pi(d_c^2 - d_g^2)}$ if it is not.

of liquid in the container, and it was decided to experiment with six combinations, a seventh being added later:

(1) 8 oz. kali bottle and gas tube, $1\frac{3}{8} \times \frac{5}{16}$ in.
(2) 8 oz. kali bottle and gas tube, $3 \times \frac{1}{2}$ in.
(3) 8 oz. kali bottle and gas tube, $4 \times \frac{3}{4}$ in.
(4) $7 \times 1\frac{1}{2}$ in. test-tube and gas tube, $1\frac{3}{8} \times \frac{5}{16}$ in.
(5) $7 \times 1\frac{1}{2}$ in. test-tube and gas tube, $3 \times \frac{1}{2}$ in.
(6) $7 \times 1\frac{1}{2}$ in. test-tube and gas tube, $4 \times \frac{3}{4}$ in.
(7) 6 oz. medicine bottle and gas tube, $3 \times \frac{1}{2}$ in.

If a large size of gas tube is important, then the contrast between groups 1 and 4 on the one hand and groups 3 and 6 on the other should tell; if a large ratio of diameter of gas tube to diameter of container is important, then $7 \times 1\frac{1}{2}$ in. test-tubes should do better than 8 oz. kali bottles, with the same size of gas tube.

We have also mentioned the 'fit' of the gas tube against the bottom of the container. When a $2 \times \frac{3}{8}$ in. gas tube is inverted in a $6 \times \frac{5}{8}$ in. test-tube the mouth of the gas tube fits closely to and almost covers the rounded base of the test-tube. Further, the gas tube can only depart very slightly from the vertical position, and the close contact between the mouth of the gas tube and the rounded base of the test-tube cannot be broken. The same applies to a $4 \times \frac{3}{4}$ in. gas tube in a $7 \times 1\frac{1}{2}$ in. test-tube. When a $3 \times \frac{1}{2}$ in. gas tube is used in a $7 \times 1\frac{1}{2}$ in. test-tube the gas tube inclines at a greater angle, and the contact between the mouth of the gas tube and the base of the test-tube, though close, is not quite so close-fitting.

The base of a kali bottle has a greater area and is slightly convex, and close contact between the mouth of the gas tube and the base of the container is impossible. The $4 \times \frac{3}{4}$ in. gas tube sometimes remains vertical or inclines at an angle of 75°, while the $3 \times \frac{1}{2}$ in. gas tube leans at an angle of 60°. In the case of the 6 oz. medicine bottle this angle is decreased to 55°. In both these last cases there is no real contact between the mouth of the gas tube and the base of the container. One point of the lip of the gas tube touches the side of the container and the diametrically opposite point touches the base, and apart from this there is no contact between the base and the gas tube. If the fit is important then groups 5 and 6 should give more positives than groups 2 and 3, and group 7 should give a low number of positives. Group 7 is a combination common in public health laboratories, where both pieces of equipment are regular stock.

In groups 1 and 4 the gas tube was suspended by a thread with the mouth just touching the bottom of the container.

Lay-out

Ten units were put up for each of the above groups and filled with 50 ml. of D.S. lactose broth (Min. Health, 1939). Into each was poured 50 ml. of distilled water which had been inoculated with 0·1 ml. of a suspension of

Bact. coli estimated to contain 5 cells per ml. The units were incubated 72 hr. at 37° C. and inspected each day.

A common criterion of a positive result is to demand that at least the round end of the gas tube be filled. Mackenzie (1940) notes also the escape of gas at the free surface of the culture and the presence of froth, and states that in doubtful cases the tapping of the tube with a ruler will often bring about an escape of imprisoned gas from the depths of the culture.

In this experiment the length of the gas bubble was measured each day, but it was noted that escape of gas from the surface ('frothing') was clearly present at the 24th hour in all cultures which then or later showed a gas bubble.

Technique

Bact. coli *suspension.*

The culture was a non-motile *Bact. coli* obtained from Lister Institute, indole+, M.R.+, V.P.−, citrate −.

The suspension was standardized to Welcome opacity tube no. 3, 0·1 ml. transferred to 100 ml. saline, repeated, 0·2 ml. transferred to 100 ml., and this third bottle then contained about 5 cells per ml. This gives about 25×10^8 viable cells per ml. in the suspension matched to Welcome's tube no. 3.

The population was checked by plate counts and shown to be remarkably near expectation. The following plates were prepared on skim milk agar (Min. Agric. 1934):

Final suspension: (i) 15 plates of 1 ml.
(ii) 3 plates of 5 ml.
Second dilution: 5 plates of 1 ml. of a 1/10 dilution.

In the case of (ii), 6 ml. of special agar was used; this agar combines the same ingredients in 600 ml. that ordinary agar has in 1000 ml. Despite this, the plates did not set well and could not be inverted.

Each unit was numbered, 1–10 for group 1, 11–20 for group 2, etc., up to 70. A random order was obtained from Fisher & Yate's tables (1938) by working down each column in turn and writing down the numbers 1–70 as they occurred. A fresh set was prepared for each experiment, working steadily through the tables.

The suspension was prepared, standardized and diluted and quantities of 0·1 ml. inoculated into 4 oz. bottles (milk bottle shape), each containing 50 ml. of sterile distilled water sterilized at least 48 hr. previously. These bottles were numbered 1–70 and were inoculated in the random order determined as above. Next the inoculated water was poured into the units, units being inoculated in the random order previously determined. One worker inoculated the bottles of water which an assistant afterwards shook. Finally the assistant passed the lactose tubes, in the correct order for inoculation, to the operator, who added the already inoculated distilled water.

Meantime another worker prepared the required plate cultures. The whole

operation took under 45 min. from the time the saline was poured on to the slant to the inoculation of the last bottle. Random inoculation was used, because it was thought that opening the culture some sixty times gave a risk of contamination in the later inoculated water bottles.

All tubes which showed reaction but no gas after 72 hr. incubation were checked. Of 1110 inoculations six were contaminated with micrococci. Some tubes which later showed gas showed no gas when inspected at 24 hr. All such tubes yielded a coliform strain with reactions as for the stock culture.

Biological factors

Age of culture.

It is known that gas production in *Bact. coli* varies with the medium, the peptone, etc. In the presence of a vigorous strain of *Str. lactis* there may be no gas production (Hassouna & Allen, 1939). The results might also be affected by previous growth conditions as well as age of the culture.

At the start of the experiments we were doing two experiments per week. The culture was started on Friday, and on Tuesday when this was 4 days old two slants were made from it, one of which was used to prepare a suspension on Wednesday when 24 hr. old and the other was carried till Friday (3 days old), when two slants were made as on Tuesday. The culture was thus a 24 hr. old growth from a culture which itself was either 3 or 4 days old. This system was followed for Exps. 1–10. For Exps. 11–14 we followed the same time table as regards experiments, but the culture, instead of being carried for 3 or 4 days and ending with a 24 hr. interval, was reinoculated every day.

It was thought that the low number of positives in Exps. 11–14 might be due to too frequent transfer of the culture and for the remaining experiments a new time table was introduced. The culture for each experiment was prepared initially 1 week before it was used, and was transferred on the third day from then and again on the sixth and was thus 24 hr. old when the suspension was made for experiment on the seventh day, on which day the culture was also reinoculated to carry on for the next week. These last experiments, 15–20, were carried out at the rate of three experiments per week. The experimental days were Friday, Saturday and Monday, and in order to observe the culture schedule just outlined and to put each experiment on the same footing we carried three series of cultures, one series for each experimental day. The Monday experiments were with a culture initially started on Monday and subcultivated as outlined, the Friday experiments were made with a culture started on Friday, and so on, thus enabling three experiments per week to be carried on indefinitely while the cultural conditions were exactly parallel for each and every experiment.

It was hoped to do 30–40 experiments and also try out combinations in smaller test-tubes, i.e. $6\frac{5}{8}$ in. test-tube with $1\frac{3}{8} \times \frac{5}{16}$ in. or $2 \times \frac{3}{8}$ in. gas tube and $6\frac{3}{4}$ in. test-tube with $2 \times \frac{1}{2}$ in. gas tube, but war-time difficulties in peptone

supply have prevented this. The data so far obtained have been analysed statistically. Part II deals with this analysis.

PART II. THE STATISTICAL ANALYSIS OF THE RESULTS

(1) *Influence of tube and container size on the percentage of positives*

The main object of the experiment was to study the influence of tube and container size on the percentage of tubes giving a positive reaction.

Table 1. *Number of positives out of* 10

Exp. no.	6 oz. medicine bottle gas-tube size $3 \times \frac{1}{2}$ in.	8 oz. kali bottle Gas-tube size $1\frac{3}{8} \times \frac{5}{16}$ in.	8 oz. kali bottle Gas-tube size $3 \times \frac{1}{2}$ in.	8 oz. kali bottle Gas-tube size $4 \times \frac{3}{4}$ in.	$7 \times 1\frac{1}{2}$ in. test-tube Gas-tube size $1\frac{3}{8} \times \frac{5}{16}$ in.	$7 \times 1\frac{1}{2}$ in. test-tube Gas-tube size $3 \times \frac{1}{2}$ in.	$7 \times 1\frac{1}{2}$ in. test-tube Gas-tube size $4 \times \frac{3}{4}$ in.
1	.	1	2	0	0	3	4
2*	.	.	.	.	.	.	.
3	.	1	1	1	3	1	4
4	.	4	2	4	1	1	3
5	.	2	0	1	2	2	5
6	.	2	7	2	2	2	4
7	.	2	1	2	1	4	4
Total		12/60 (20%)	13/60 (21·7%)	10/60 (16·7%)	9/60 (15·0%)	13/60 (21·7%)	24/60 (40%)
8	.	1	1	1	1	3	2
9	.	3	4	2	2	1	3
10	1	3	2	4	3	3	6
11	4	2	4	1	1	0	0
12	2	2	1	3	2	4	6
13	1	1	1	2	0	2	1
14	4	5	5	5	3	5	5
15	0	1	1	1	0	2	0
16	1	0	1	2	2	2	2
17	1	3	4	5	1	1	3
18	2	3	4	1	3	8	6
19	1	0	0	4	0	2	1
20	1	0	1	2	0	3	1
Total	18/110 (16·4%)	24/130 (18·5%)	29/130 (22·3%)	33/130 (25·4%)	18/130 (13·8%)	36/130 (27·7%)	36/130 (27·7%)
No. 18 omitted		21/120 (17·5%)	25/120 (20·8%)	32/120 (26·9%)	15/120 (12·5%)	28/120 (23·3%)	30/120 (25·0%)

* Exp. 2: population excessive, apparently an error in diluting.

Table 1 gives the number of tubes positive out of ten in each of nineteen experiments, for each type of apparatus. In Exps. 1–7, unfortunately, a double mark pipette was used as though it had been a blow-out pipette. This means that instead of inoculating 0·1 ml. a rather greater quantity was inoculated, which would be about 0·12 ml., but which would vary from one experiment to the next, depending on how near the lower mark was to the tip of the pipette. Despite this the numbers of positives for the different types of apparatus in any one experiment are comparable with one another because the inoculations in any one experiment were made with the same pipette. In Exps. 8–20 the correct amount of 0·1 ml. was inoculated. For this reason the results for the first seven experiments have been analysed separately.

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In point of fact Exp. 2 failed, so there were only six experiments to analyse. In the event, as we shall see, there was no significant difference between the six experiments in the resulting percentage of positives, so the amount inoculated may be taken as 0·12 ml., within the limits of experimental error.

Prima facie, the simplest method of analysis is to calculate the percentage of positives over all experiments together for each type of apparatus, and to test whether these percentages differ significantly by a χ^2-test. This is, however, to some extent invalidated if the actual experimental error for any one experiment with a particular type of apparatus exceeds the theoretical value postulated by the binomial distribution, and more information can be obtained from an analysis of variance which eliminates the difference between experiments and enables the effect of tube size and container size as well as their interaction to be tested. This will be dealt with in due course.

The percentage of positives is also given in Table 1 for Exps. 1–7 and 8–20 combined. Taking first Exps. 1–7 the values of χ^2 is 13·9 with 5 degrees of freedom. This is significant ($P=0{\cdot}016$). The significance is due to the excess of positives in the combination of the $7\times1\frac{1}{2}$ in. test-tube with the $4\times\frac{3}{4}$ in. gas tube. For Exps. 8–20 the value of χ^2 is 11·2 with 6 degrees of freedom. This is not significant at the 5% level (5% point = 12·6), but there is a suggestion that the last type of apparatus maintains its superiority.

We now proceed to the analysis of variance to see if this confirms our first impression. At any one level p of the true proportion positive, the variation in parallel sets of tube will be of binomial type; accordingly, the sampling variance will be different at different levels of p. In applying analysis of variance methods we require the same sampling variance at all levels of p; it is accordingly customary to use a transformed variate ϕ defined by

$$p=\sin^2\phi.$$

Since
$$\phi=\sin^{-1}\sqrt{p}$$

and
$$\delta\phi=\delta p/2\sqrt{\{p(1-p)\}},$$

the sampling variance of ϕ is given by

$$V(\phi)=\frac{V(p)}{4pq}=\frac{1}{4n}\text{ approx.},$$

where n is the number of tubes. This is independent of p. If ϕ is measured in degrees

$$V(\phi)=\left(\frac{180}{\pi}\right)^2\frac{1}{4n}=\frac{8100}{\pi^2 n}=\frac{820{\cdot}7}{n}. \qquad (1)$$

This is the theoretical error variance, and the error variance obtained from the analysis may be compared with this as a check on the theoretical assumptions. Fisher & Yates (1938) give a table for transforming p to ϕ and vice versa.

To illustrate the procedure Table 2 shows the values of ϕ obtained for Exps. 1–7.

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Table 2. *Values of* $10p$ *and of* ϕ *in degrees*

	8 oz. kali bottle Gas tube			7 × 1½ in. test-tube Gas tube		
Exp.	$1\frac{3}{8} \times \frac{5}{16}$ in.	$3 \times \frac{1}{2}$ in.	$4 \times \frac{3}{4}$ in.	$1\frac{3}{8} \times \frac{5}{16}$ in.	$3 \times \frac{1}{2}$ in.	$4 \times \frac{3}{4}$ in.
1	1	2	0	0	3	4
	18·4	26·6	0	0	33·2	39·2
3	1	1	1	3	1	4
	18·4	18·4	18·4	33·2	18·4	39·2
4	4	2	4	1	1	3
	39·2	26·6	39·2	18·4	18·4	33·2
5	2	0	1	2	2	5
	26·6	0	18·4	26·6	26·6	45·0
6	2	7	2	2	2	4
	26·6	56·8	26·6	26·6	26·6	39·2
7	2	1	2	1	4	4
	26·6	18·4	26·6	18·4	39·2	39·2

The analysis of variance is as follows:

Exps. 1–7 (*less* 2). *Analysis of variance*

	Sum of squares		Degrees of freedom		Mean square		Variance ratio	
Experiments	729·448		5		145·890		1·138	
Treatments:								
Gas tube	311·002	1352·650	2	5	155·501	270·530	1·213	2·113
Container	219·040		1		219·040		1·709	
Interaction	822·608		2		411·304		3·208	
Error	3204·830		25		128·193			
Total	5286·928		35					
Theoretical error variance					82·07			
5% values of variance ratio							2·6 for 5 and 25 D.F. 3·4 for 2 and 25 D.F. 4·2 for 1 and 25 D.F.	

The only effect which approaches significance at the 5% level is the interaction between gas-tube size and container size. This suggests that the difference between the percentage of positives when the bottle and the test-tube are used is different for the different sizes of gas tube. The mean values of ϕ and the corresponding percentages are as follows:

Exps. 1–7

		Gas tube			
		$1\frac{3}{8} \times \frac{5}{16}$ in.	$3 \times \frac{1}{2}$ in.	$4 \times \frac{3}{4}$ in.	
Bottle:	ϕ	25·9	24·4	21·5	
	$100p$	19·1	17·0	13·4	
Test-tube:	ϕ	20·5	27·1	39·2	
	$100p$	12·2	20·1	40·0	
Diff. (test-tube – bottle):					
	ϕ	−5·4	+2·7	+17·7	S.E. 6·5
	$100p$	−6·9	+3·1	+26·6	

Here again we notice the large difference between test-tube and bottle in the last combination; being the greatest out of three differences, this also just falls short of the 5% significance level.

The mean value of ϕ for all treatments is 26·5, and the corresponding value of the percentage positive is 19·9. These values of $100p$ are somewhat less

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than those given by Table 1, because a different process of averaging has been employed (cf. the difference between the arithmetic and geometric mean).

The error variance is slightly greater than the theoretical value calculated from (1) as one might expect. Comparison with it yields a χ^2 of 39·05, which is just significant for 25 degrees of freedom (5% point = 37·65). This is why the analysis of variance just fails to give a significant result, although the preliminary analysis did so. The result of the analysis tends to confirm the conclusion of the preliminary analysis that the combination of the test-tube with the $4 \times \frac{3}{4}$ in. gas tube gives the largest percentage of positives, it also suggests that the advantage which the test-tube has over the bottle is greatest with the $4 \times \frac{3}{4}$ in. gas tube.

From the analysis of variance of Exps. 8–20, no. 18 was omitted. This was because the plate count showed the experiment to be anomalous (see p. 189). The combination of 6 oz. medicine bottle with $3 \times \frac{1}{2}$ in. test-tube which was outside the original scheme of the experiment was omitted.[1]

The analysis is as follows:

Exps. 8–20. *Analysis of variance*

	Sum of squares		Degrees of freedom		Mean square		Variance ratio	
Experiments	6142·845		11		558·440		5·207	
Treatments:								
Gas tube	1194·568	1409·591	2	5	597·284	281·918	5·569	2·628
Container	60·500		1		60·500		0·564	
Interaction	154·523		2		77·261		0·720	
Error	5899·049		55		107·255			
Total	13451·485		71					
Theoretical error variance					82·07			
5% values of variance ratio							2·0 for 11 and 55 D.F. 2·4 for 5 and 55 D.F. 3·2 for 2 and 55 D.F. 4·0 for 1 and 55 D.F.	

Here the difference between the results of the different experiments, of course, is highly significant (the variance ratio exceeds the 0·1% point of 3·4) and the treatment differences are striking. The latter are due to the differences between the gas tubes (for which the 1% variance ratio is 5·0). There is no significant difference between the two types of container and no significant interaction.

The mean values of ϕ and the corresponding percentages are as follows:

Exps. 8–20

		Gas tube			
		$1\frac{3}{8} \times \frac{5}{16}$ in.	$3 \times \frac{1}{2}$ in.	$4 \times \frac{3}{4}$ in.	
Bottle:	ϕ	21·1	25·0	30·3	
	$100p$	13·0	17·8	25·4	
Test-tube:	ϕ	16·8	27·2	26·8	
	$100p$	8·4	21·8	20·3	
Mean:	ϕ	18·9	26·1	28·5	S.E. 2·1
	$100p$	10·5	19·4	22·7	

[1] The mean value of ϕ for this type of apparatus is 22·0 with S.E. 3·1, the corresponding percentage positive being 14·0. This does not differ significantly from the value 26·1 ($100p = 19·4$) for the mean of Exps. 8–20 with the same type of gas tube and the other two types of container.

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Here the combination of bottle with $4 \times \frac{3}{4}$ in. gas tube appears to give the largest percentage of positives, but this percentage does not differ significantly either from the corresponding result with the test-tube or from that with the $3 \times \frac{1}{2}$ in. gas tube; neither do the mean results for the $3 \times \frac{1}{2}$ and $4 \times \frac{3}{4}$ in. gas tubes differ significantly. *It is the low percentage of positives with the* $1\frac{3}{8} \times \frac{5}{16}$ *in. gas tube which is the striking feature of the results*, and this is also suggested by Exps. 1–7. This is the only indisputably significant result from the whole series of experiments. In the second series the error variance is not significantly in excess of its theoretical value ($\sqrt{(2\chi^2)} = 12{\cdot}0$, $\sqrt{(2n-1)} = 10{\cdot}5$).

Table 3. *Plate count results*

	First series (15 plates with 1 ml.) 5 colonies per plate assumed		Second series (3 plates with 5 ml.) 25 colonies per plate assumed			Third series (5 plates with 1 ml. 50 times as strong) 250 colonies per plate assumed		
Exp.	Mean per plate	χ^2 (4 D.F.)	Mean per plate	Mean per ml.	χ^2 (2 D.F.)	Mean per plate	Mean per ml.	χ^2 (4 D.F.)
1	4·33	4·46	21·00	4·20	0·09			
2*	—	—	—	—	—			
3	5·47	12·76	29·33	5·87	4·45			
4	7·07	12·87	†—	—	—			
5	5·87	12·23	23·00	4·60	0·09			
6	4·87	4·88	24·00	4·80	0·58			
7	5·40	7·33	15·33	3·07	0·17	195·8	3·92	2·97
8	3·33	13·00	16·33	3·27	5·67	141·8	2·84	3·35
9	3·80	10·11	15·33	3·06	1·61	166·2	3·32	5·38
10	6·20	6·19	22·67	4·43	0·56	238·2	4·76	1·19
11	5·40	13·63	28·33	5·67	4·12	191·4	3·83	3·96
12	5·20	15·84	28·33	5·67	1·44	198·0	3·96	1·98
13	6·67	8·90	33·00	6·60	0·54	296·8	5·94	0·16
14	7·47	9·34	43·33	8·67	0·11	265·4	5·31	0·95
15	5·53	12·24	21·33	4·27	3·13	186·4	3·73	0·95
16	4·40	8·55	30·67	6·13	0·41	290·4	5·81	1·80
17	6·53	5·16	32·33	6·47	4·60	281·4	5·63	4·90
18	[10·13]	[3·62]	[46·33]	[9·27]	[1·91]	[512·6]	[10·25]	[2·51]
19	6·87	11·90	36·33	7·27	0·13	287·4	5·75	0·98
20	7·40	8·87	31·67	6·33	2·80	299·0	5·98	4·05
All experiments (18 omitted):								
Mean	5·66			5·32			4·68	
Total χ^2		178·26			30·50			32·62
$\sqrt{(2\chi^2)} - \sqrt{(2n-1)}$		−3·55			−0·25			−2·07

* Exp. 2: population excessive, apparently an error in diluting.

† One plate out of the three contaminated.

(2) *The plate counts*

The aim was to obtain a plate count of 5 colonies per ml. for the dilution which was used for the coliform tests. Three series of plate counts were carried out: (i) fifteen plates with 1 ml. of the above dilution, (ii) three plates with 5 ml., (iii) five plates with 1 ml. of a dilution 50 times as strong. The mean counts for each experiment and the values of χ^2 are shown in Table 3.

The general means will shortly be shown not to differ significantly from 5. The values of χ^2 are on the whole, somewhat *subnormal*, in the first and third series significantly so. The agreement between parallel plates is, on the average, closer than one would expect by chance on the basis of a Poisson

distribution of colony numbers. This phenomenon has been noticed before. Fisher *et al.* (1922) called attention to it in connexion with bacterial counts of soil organisms and attributed it to a defect in the medium.

The count in Exp. 18 is clearly too high, and has been omitted from the subsequent statistical analysis. It is clear that something abnormal occurred in this experiment.

Table 4. *Analysis of variance for plate counts*

Assumed no. of colonies per plate		Sum of squares	Degrees of freedom	Mean square	Variance ratio
		Exps. 1–7			
5	Between experiments	65·43	5	13·09	3·49
	Within experiments	315·07	84	3·75	($P<0·01$)
	Total	380·50	89		
	General mean	5·50	S.E. $\sqrt{(13·09/90)}=0·38$		
25	Between experiments	308·40	4	77·10	5·09
	Within experiments	151·33	10	15·13	($P<0·05$)
	Total	459·73	14		
	General mean: per plate	22·53	S.E. $\sqrt{(77·10/15)}=2·27$		
	per ml.	4·51	S.E.	0·45	
250	Between experiments	Only one experiment			
	Within experiments	580·8	4	145·2	
	General mean: per plate	195·8	S.E. $\sqrt{(16285/5)}=57·1$		
	per ml.	3·92	S.E.	1·14	
		Exps. 8–20			
5	Between experiments	307·60	11	27·96	6·79
	Within experiments	691·60	168	4·12	($P<0·001$)
	Total	999·20	179		
	General mean	5·73	S.E. $\sqrt{(27·96/180)}=0·39$		
25	Between experiments	2212·31	11	201·12	7·65
	Within experiments	631·33	24	26·31	($P<0·001$)
	Total	2843·64	35		
	General mean: per plate	28·31	S.E. $\sqrt{(201·12/36)}=2·36$		
	per ml.	5·66	S.E.	0·47	
250	Between experiments	179137	11	16285	115
	Within experiments	6816	48	142	($P<0·001$)
	Total	185953	59		
	General mean: per plate	236·9	S.E. $\sqrt{(16285/60)}=16·5$		
	per ml.	4·74	S.E.	0·33	
		Whole series			
	Weighted mean per ml.	5·17	S.E.	0·17	

For Exps. 1–7 and 8–20 omitting no. 18, analysis of variance within and between experiments yielded the results given in Table 4. The subnormal variance of plates from the same experiment is exhibited clearly. The expected values on the basis of the Poisson distribution are 5, 25 and 250 respectively. The observed values are 3·99, 23·02, 142·2. The reduction is significant in the first and third series. It is only material in the last series where the standard deviation is about three quarters of that expected.

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There are significant differences between individual experiments in each case, but owing to the inter-experimental variation the means for each series and the general mean do not differ significantly from 5 colonies per ml. It is clear that the density of 5 colonies per ml. aimed at was closely realized.

It may be asked whether fifteen plates with 1 ml. or three plates with 5 ml. give the more accurate result. If the distribution of colony numbers is exactly a Poisson distribution there is of course no difference. Actually the variance of the mean number of organisms per ml. is $(3{\cdot}99/15) = 0{\cdot}266$ for an experiment of the first kind and $(23{\cdot}02/75) = 0{\cdot}307$ for one of the second. The variance ratio 1·15 does not differ significantly from unity at the 5 % level. Thus an advantage in favour of the first method is not established.

(3) *Relation between the plate counts and the presumptive coliform tests*

In the presumptive coliform tests the overall percentage of tubes positive is 22·5 % for Exps. 1–7. For Exps. 8–20 it is as follows:

		Medicine bottle and $3 \times \frac{1}{2}$ in. gas tube	
		Included	Excluded
Exp. 18:	Included	21·8	22·6
	Excluded	20·4	21·0

It is clear that the average percentage of positives is about 20 %. With 0·5 organism in 0·1 ml. (Exps. 8–20) we should expect 100 $(1-e^{-0{\cdot}5}) = 39$ % of tubes to be positive. With 0·6 (Exps. 1–7) we should expect 45 %. It is clear that the percentage of positives is about half that to be expected from the plate counts. If it took two original organisms per tube to cause a positive reaction we should expect 100 $(1-1{\cdot}5e^{-0{\cdot}5}) = 9$ % of positives in the first case and 12 % in the second, which are too small. It seems more likely that the medium used in the coliform tests is less favourable to the organisms than that used in the plate count, so that a proportion of the original organisms die prior to multiplication.

(4) *Influence of shape and size factors*

We cannot in these experiments distinguish between the importance of the absolute size of the gas tube, and of the ratio of the volume of the gas tube to the total volume of liquid; for the total volume of liquid (100 ml.) is the same in every treatment combination. Size of gas tube, however, whether absolute or relative, is of importance, for the $1\frac{3}{8} \times \frac{5}{16}$ in. gas tube gave a smaller percentage of positives than the others.

If ratio of diameter of gas tube to diameter of container were important we should have expected a significant difference between the average results for the 8 oz. kali bottles and the $7 \times 1\frac{1}{2}$ in. test-tubes, but this was not found.

We cannot examine the importance of length of gas tube per se, for the longer gas tubes are also the larger ones. It is clearly worth while giving some thought to the problem of what treatment combinations are likely to be most informative in future experiments.

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Part III. Appendix. Two further experiments

Since the completion of the previous experiments, two further experiments have been performed: (1) a comparison of two different sizes of gas tube, and (2) a comparison of rimmed and rimless tubes.

(1) *Comparison of* $2 \times \frac{3}{8}$ *in. and* $1\frac{3}{8} \times \frac{5}{16}$ *in. gas tubes*

The experiment was designed to disclose any differences in the number of positives in the presumptive coliform test, using $6 \times \frac{5}{8}$ in. test-tubes with either $2 \times \frac{3}{8}$ or $1\frac{3}{8} \times \frac{5}{16}$ in. gas tubes. The former combination is favoured by the Ministry of Agriculture (1934) while the smaller gas tube is the size approved by the Ministry of Health (1937).

In each experiment 40 tubes of each combination were inoculated with 1 ml. of a suspension of *Bact. coli* estimated to contain 1 cell per 2 ml. and the tubes incubated 3 days at 37° C. Results were recorded at 24 and 72 hours; acid and gas being accepted as a positive reaction.

Technique.

The medium was made in accordance with the formula of the Ministry of Health (1937), but was adjusted and coloured with litmus (Min. Agric. 1934). All test-tubes and gas tubes were checked for size and the suspension was prepared as in previous experiments. These showed that a suspension matched to opacity tube no. 3 contained 25×10^8 viable units according to plate counts. In the present experiments this suspension was diluted as follows:

First dilution bottle. 0·1 ml. of suspension into 100 ml. of saline.
Second dilution bottle. 0·1 ml. of no. 1 into 100 ml. of saline.
Third dilution bottle. 2 ml. of no. 2 into 100 ml. of saline.
Fourth dilution bottle. 1 ml. of no. 3 into 100 ml. of saline.

Finally 1 ml. of no. 4 bottle was inoculated into each tube. The population was checked by preparing five plates each containing 1 ml. of 1 in 10 dilution of bottle no. 2.

If we disregard the error between 100 ml. and 100·1 ml. but recognize the additional 2 and 1 ml. in bottles 3 and 4 this gives an expected population of 0·485 per ml.

A fresh slant and new suspension was prepared for each experiment, but all were carried through on the same morning, the average time being $\frac{3}{4}$ hr. per experiment. The two types of apparatus were inoculated alternately, first a tube with a $2 \times \frac{3}{8}$ in. gas tube and then a tube with a $\frac{5}{16}$ in. gas tube and so on until the 80 tubes had been inoculated. Each tube was shaken four times after inoculation by rapidly twisting the wrist so that the base travelled about 1 in. either side of the vertical.

Results.

Measurements of the length of the gas column were not made, but all tubes which were positive gave a clear reaction at 24 hr. The results were as follows:

Number of positives out of 40 *after* 24 *hr. incubation*

Exp.	2 × ⅜ in. gas tube	1⅜ × ⅜ in. gas tube
1	19	16
2	19	18
3	16	23
4	24	13
5	17	23
6	12	19
	107/240 = 44·6 %	112/240 = 46·7 %

The difference in the percentage of positives 2·1 % has a standard error of 4·6 % and is clearly not significant. The analysis of variance of $\phi = \sin^{-1}\sqrt{p}$ and is as follows:

	Sum of squares	Degrees of freedom	Mean square
Experiments	54·99	5	11·00
Sizes of gas tube	4·20	1	4·20
Error	278·27	5	55·65
Total	337·46	11	30·68
Theoretical error variance (820·7/40)			20·52

There are no significant differences between experiments or between the two tube sizes and the total variance is in good agreement with its theoretical value ($\chi^2 = 16{\cdot}4$, 5 % point = 19·7).

Five plate counts were made in each experiment with the same technique as before. The mean results with an expected count of 250 colonies per plate were:

Exp. 1	284·8	4	256·8	
Exp. 2	323·6	5	325·0	
Exp. 3	308·4	6	321·0	
All experiments			303·3	S.E. 11·2

The analysis of variance between and within plates is as follows:

	Sum of squares	Degrees of freedom	Mean square	Variance ratio
Between experiments	18,634	5	3727	9·8
Within experiments	9,114	24	380	($P < 0{\cdot}001$)
	27,748	29		

These are significant differences between experiments and the mean is higher than the 250 expected.

The mean plate count corresponds to 0·607 organism per tube; the corresponding percentage of positive tubes is $100\,(1 - e^{-0{\cdot}607}) = 45{\cdot}5\,\%$, in remarkably good agreement with that observed.

(2) *Comparison of rimmed and rimless gas tubes*

Twenty-seven tubes of each type were used in each experiment with 6 × ⅝ in. test-tubes and 2 × ⅜ in. gas tubes. Technique, preparation of suspension

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and other details were the same as in the previous experiment. The results are given below:

Number of positives out of 27 *after* 72 *hr. incubation*[1]

Exp.	Rimmed	Rimless
1	13	13
2	13	9
3	11	10
4	7	13
5	10	9
6	8	9
	62/162 = 38·3 %	63/162 = 38·9 %

It is clear that there is no significant difference between the rimmed and rimless tubes. The analysis of variance of $\phi = \sin^{-1}\sqrt{p}$ and is as follows:

	Sum of squares	Degrees of freedom	Mean square
Experiments	112·19	5	22·44
Rimmed or rimless	0·56	1	0·56
Error	133·00	5	26·60
	245·75	11	22·34
Theoretical error variance (820·7/27)			30·40

Again there are no significant differences between experiments or between the two tube sizes and the total variance is in good agreement with its theoretical value ($\chi^2 = 8{\cdot}1$ with 11 D.F.).

Five plate counts were made in each experiment as before. The mean results were

Exp. 1	344·6	4	286·2	
Exp. 2	268·8	5	287·8	
Exp. 3	251·4	6	245·0	
All experiments			280·6	S.E. 14·6

The analysis of variance between and within plates is as follows:

	Sum of squares	Degrees of freedom	Mean square	Variance ratio
Between experiments	32192	5	6438	16·5
Within experiments	9365	24	390	($P < 0{\cdot}001$)
	41557	29		

These are significant differences between experiments and the mean is slightly higher than the 250 expected.

The mean plate count corresponds to 0·561 organism per tube; the corresponding percentage of positive tubes is 42·9 %, again very close to the 38·6 % actually observed.

[1] The results after 24 hr. were the same, except in Exps. 4 and 5 which gave respectively 6 positives with rimmed tubes and 8 positives with rimless instead of 7 and 9.

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PART IV. DISCUSSION AND SUMMARY

1. The primary aim of the experiment was to find out if certain combinations of container and gas tube give a greater proportion of positive results than others (with the same strength of inoculation) and if possible to elucidate the causes of any differences between combinations.

The earlier results indicated that the best combination was a $7 \times 1\frac{1}{2}$ in. test-tube with a $4 \times \frac{3}{4}$ in. gas tube. Later experiments failed to maintain this preference but did show a definite disadvantage in using a very small gas tube. Whether this is due to the absolute smallness of the gas tube or the smallness of its volume relative to the total amount of liquid used cannot be decided, for the latter was the same in all combinations. This was the only shape or size factor that was definitely influential on the evidence of these experiments. Yet if size of gas tube is intrinsically important the absence of reactions with acid but no gas is puzzling.

Attempts to break down the points involved in changes in shape and size of gas tube and container into a number of simple factors showed that several considerations might be involved, too many to cover in one experimental lay-out; but by including extreme combinations it was hoped that some indication might be obtained of the more important factors. With the new and successful combination of $7 \times 1\frac{1}{2}$ in. test-tube with $4 \times \frac{3}{4}$ in. gas tube, the gas tube holds 22 % of the total volume of medium plus inoculum, the same proportion as the standard $6 \times \frac{5}{8}$ in. test-tube and $1\frac{3}{8} \times \frac{5}{16}$ in. gas tube. This seems to be in support of standard practice if it is relative volumes that are important.

2. The analysis of plate counts shows that the opacity method, with the strain employed, gives complete control of the population and the three methods of assessing the plate count were in good agreement with one another.

The χ^2 values were as a rule *subnormal*. This result excludes the possibility of any lack of control of the population in any one set of plates, but the reason for the subnormality is of some interest. It may plausibly be attributed to the factor of competition among developing colonies for the nutrient medium available so that the chance of development of a colony is smaller when a large number of others are present than when there are only a few. A toxic emanation from a colony which excluded the formation of another colony within a certain distance of it would have the same effect.

3. In the main experiment the percentage of positive tubes was about half (20 % as against 45 %) that to be expected from the plate counts on the usual Poisson hypothesis; or looking at the matter the other way the mean number of organisms per tube (0·22) deduced from the percentage of positives was about half the 0·5 expected from the plate counts. We have found

no reason for this and in two subsequent experiments the agreement was satisfactory.

4. No difference was found between $2 \times \frac{3}{8}$ and $1\frac{3}{8} \times \frac{5}{16}$ in. gas tubes with a $6 \times \frac{5}{8}$ in. test-tube or between rimmed and rimless gas tubes.

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