STUDIES OF RICINOLEIC ACID AND A TURBIDIMETRIC METHOD OF EVALUATING THE BACTERICIDAL ACTION OF SOLUTIONS OF PHENOLS IN POTASSIUM RICINOLEATES

PART III—REPRODUCIBILITY OF NEPHELOMETER RESULTS

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INTRODUCTION

IN Part II of this series[1] it was shown that further investigations were necessary before the nephelometer results could be used for evaluating the bactericidal action of a bactericide. The large daily variation noted in the earlier investigations was attributed to the treatment accorded the suspension of *Bacterium coli* after the initial standardisation of the suspension. This initial suspension was mixed with a solution of phenol and then sampled at predetermined time intervals, the samples were incubated for 5 hours and their turbidities were measured on the nephelometer. The assumption that equal nephelometer readings, in two different experiments, indicated an equal number of survivors in the samples appeared incorrect and so a technique was evolved in this part of the work to make this assumption unnecessary.

REFERENCE TURBIDITY

As a direct relationship between potentiometer reading and percentage survivors was not possible it was necessary to introduce a reference turbidity which, whilst subject to the same variation as the test material, remained a reference in that it was grown from a known or standard inoculum. The principle being that if test and reference cultures were incubated under identical conditions then the number of survivors in the test could be found by direct comparison of its turbidity with an equal turbidity produced in the reference culture.

It was decided that the standard suspension prepared daily for each experiment would serve to provide this reference turbidity. However the initial suspension itself could not be used undiluted since its turbidity after 5 hours incubation would be too great to be measured on the nephelometer ; nor could a shorter period of incubation of the reference culture be used since reference and test would not then be receiving identical incubation treatments and so not allow of any turbidity comparisons. A system of diluting the initial suspension was evolved which enabled it to be used as a reference turbidity.

APPARATUS

The apparatus used was that described in Part II of this work[1] with the exception of the water-bath used for incubation in the final part of this work.

Incubation. In the initial experiments when a batch of 10 bottles of

medium had been inoculated it was transferred from the water-bath at 37°C. to an electrically heated incubator at 37° ± 0·5°C. for the 5 hour incubation period. During the experiment the door of the incubator had to be opened and closed three or four times during the first hour or 90 minutes ; because of this a careful check was carried out on the incubator temperatures on each of several days and a temperature drop of 0·8° to 1·0°C. for periods as long as 20 minutes was observed. An experiment was performed to ascertain the effects if any of these small temperature changes upon the turbidities of the cultures. The results of this experiment, summarised in Table I, clearly indicated that the temperature of incubation was very important, an observation which was supported by the consideration of the fact that the incubation period was during the initial stages of the logarithmic growth phase of the bacteria.

TABLE I

SHOWING THE EFFECT OF A SMALL CHANGE OF INCUBATION TEMPERATURE ON THE TURBIDITY AFTER 5 HOURS INCUBATION OF A SUSPENSION OF *BACTERIUM COLI*

Inoculum (measured as drops of initial suspension)	1	2	3	5	5	10	15	25
Turbidity (nephelometer reading) after incubating at 36·5°C.±0·3°C.	12 11	14 12	17 17	28 24	20 19	30 32	44 43	58 64
Turbidity (nephelometer reading) after incubating at 37·2°C.±0·2°C.	14 14	18 20	24 22	37 44	38 37	62 64	67 75	75 85

Joslyn[2] encountered a similar difficulty in a 4-hour determination of penicillin activity, and recommended the use of a constant temperature water-bath for short incubation periods. A constant temperature water-bath was designed to hold 80 culture bottles, which, when in place, were immersed up to their necks. The temperature was kept constant by means of an 11-inch bulb type toluene thermo-regulator and a hot-wire vacuum switch, which controlled the current to a 750 watt immersion heater ; efficient stirring was maintained throughout the incubation period by two 1/60th H.P. high-speed stirrers. Trials indicated that a temperature of 37° ± 0·2°C. could be maintained for 8 hours, although it was noticed that removal of the bottles individually at the end of the 5-hour incubation period caused a drop in water level in the bath and the range was increased during this final period to ± 0·5°C.

EXPERIMENTAL

The experimental details for this work were essentially the same as those outlined in Part II[2]. A further detail was the inoculation of 10 culture bottles at the start of the experiment and a further 10 bottles at the end of the experiment to give the reference turbidity. The method was as follows :—

1.—The initial suspension was prepared as before, labelled S.100 per cent. and placed in the water-bath at 20°C.

2.—10ml. of this suspension was pipetted into a bottle containing 90ml. of sterile water. This bottle was labelled R.10 per cent. shaken and placed in the water-bath at 20°C.

3.—2ml. of the suspension S.100 per cent. was pipetted into a bottle

containing 98ml. of sterile water. This bottle was labelled R.2 per cent. and also placed in the bath at 20°C.

4.—10ml. of sterile distilled water was pipetted into each of 4 sterile medication tubes and the tubes placed in the bath at 20°C.

5.—Into each of 2 tubes containing 10ml. of sterile water, 5ml. of R.10 per cent. was pipetted and the tubes returned to the water-bath, after labelling R.10 per cent.

6.—Similarly 5ml. of R.2 per cent. were pipetted into each of the two remaining tubes containing 10ml. of sterile water, the tubes labelled R.2 per cent. and returned to the bath.

7.—At a predetermined time one of the R.10 per cent. tubes was taken from the water-bath and shaken. A sterile dropping pipette was rinsed with the suspension in the tube by drawing the mixture up and down the pipette twice, the pipette was filled with the suspension and the tube discarded.

8.—A culture bottle containing 50ml. of sterile peptone broth was taken from the 37°C. water-bath and inoculated with 5 drops of the R.10 per cent. suspension of (7), after shaking to disperse, the bottle was returned to the 37°C. bath.

9.—This was repeated with 4 other culture bottles adding respectively 4, 3, 2, and 1 drops of the R.10 per cent. suspension.

10.—Repeated (7), (8), (9) with 1 of the medication tubes labelled R.2 per cent., a fresh sterile dropping pipette and 5 further culture bottles each containing 50ml. of sterile broth.

This established the reference turbidity at the beginning of the experiment, a similar procedure at the end of the experiment gave a similar

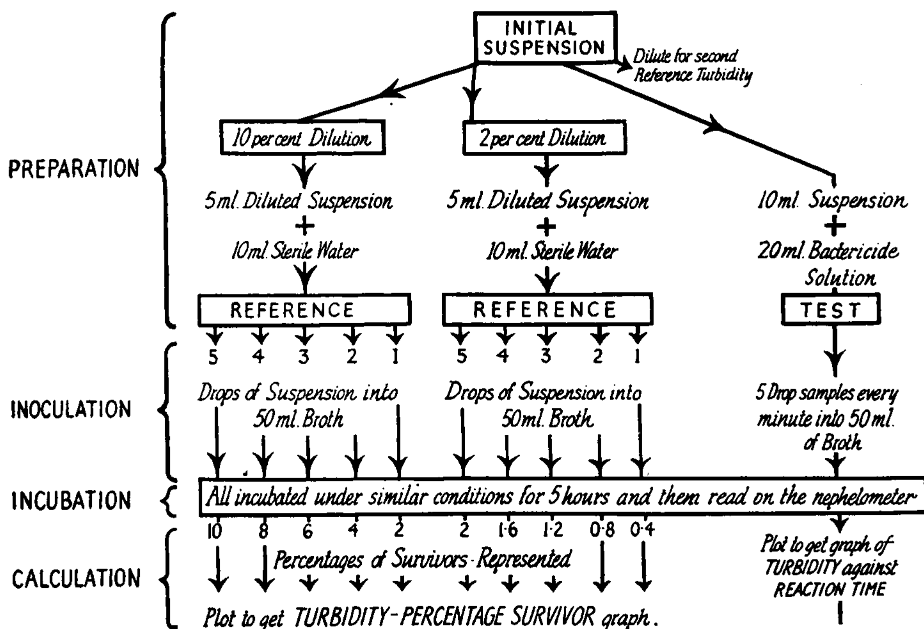

FIG. 1 Schematic diagram of a single experiment.

reference turbidity finally. Any difference in these reference turbidities indicated a variation in the culture during the inoculation stage of the experiment.

As in Part II the turbidities were read on the nephelometer after exactly 5 hours incubation.

Figure I shows a schematic diagram of a single experiment and how the data for the two graphs for each experiment were obtained.

Calculation of Results. Tables II and IV are the results obtained in a typical experiment.

TABLE II

RESULTS OF THE NEPHELOMETER READINGS OBTAINED IN A TYPICAL EXPERIMENT FROM WHICH A REFERENCE TURBIDITY CURVE WAS PLOTTED

Suspension used	R. 10 per cent.					R. 2 per cent.				
No. of drops ...	5	4	3	2	1	5	4	3	2	1
	NEPHELOMETER READINGS									
Before test ...	84	81	77	67	48	46	40	32	30	19
After test	78	77	70	56	49	40	41	31	—	20
Average	81	79	73½	61½	46		40½	31½	30	19½
Percentage of survivors	10	8	6	4	2		1·6	1·2	0·8	0·4

From the results in Table II the graph in Figure 2 was drawn using a logarithmic scale for the percentage survivors.

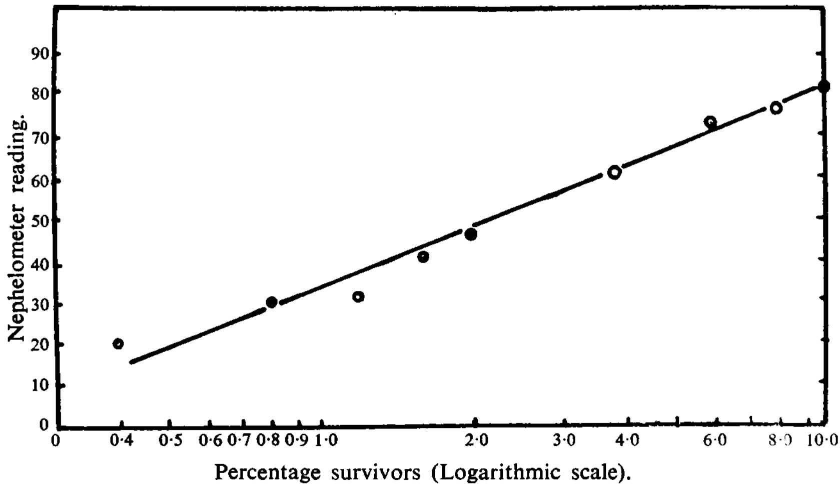

FIG. 2. Reference turbidity. Nephelometer reading plotted against the logarithm of the percentage of survivors.

From this graph another table (Table III) was compiled giving the nephelometer readings corresponding to various percentages of survivors.

In this way the reference turbidity was used to convert the nephelometer readings into percentage survivors.

TABLE III

NEPHELOMETER READINGS CORRESPONDING TO VARIOUS PERCENTAGES OF SURVIVORS, COMPILED FROM FIG. 2

Survivor Percentage ...	0·5	0·6	0·7	0·8	0·9	1·0	2·0	3·0	4·0	5·0	6·0	7·0	8·0	9·0	10·0
Nephelometer Reading ...	19	23	26	29	31	33	48	56	62	67	71	74	77	80	82

From the results of test I in Table IV a graph of nephelometer reading against reaction time was drawn (Fig. 3).

TABLE IV

RESULTS, FROM A TYPICAL EXPERIMENT, OF THE NEPHELOMETER READINGS OBTAINED USING THREE SAMPLES OF A 1 PER CENT. PHENOL SOLUTION AGAINST BACT COLI.

Reaction Time	Nephelometer Readings I	II	III
1 minute ...	100	95	B.S.
2 ,, ...	97	99	B.S.
3 ,, ...	90	89	91
4 ,, ...	89	79	80
5 ,, ...	73	75	77
6 ,, ...	64	60	62
7 ,, ...	54	41	58
8 ,, ...	41	37	49
9 ,, ...	41	24	41
10 ,, ...	30	25	28
11 ,, ...	22	19	31
12 ,, ...	26	16	22
13 ,, ...	17	17	18
14 ,, ...	13	18	31
15 ,, ...	16	15	16

B.S.—Beyond the scale of the instrument.

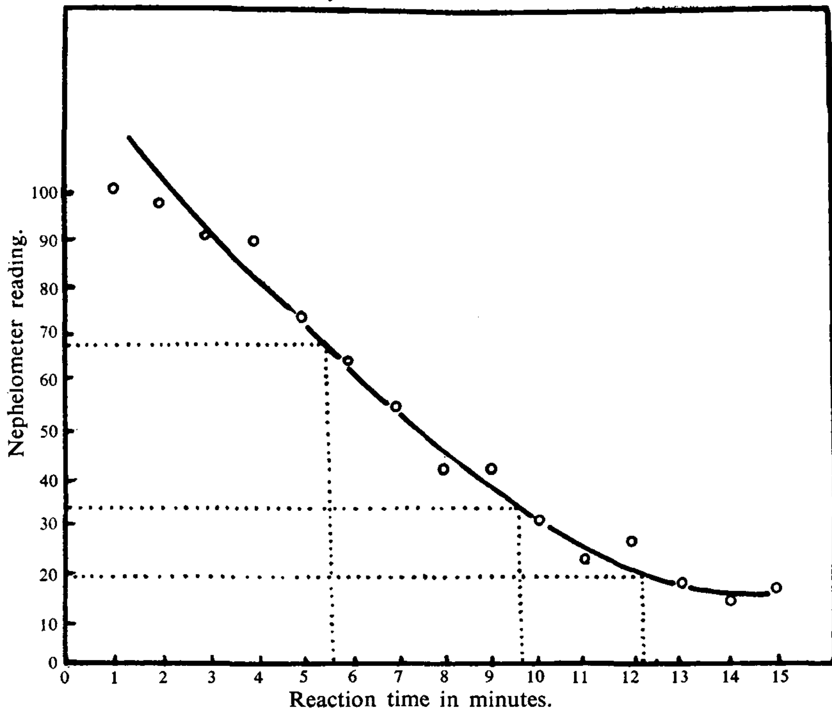

FIG. 3. Test turbidity. Showing the turbidities developed by survivors after various times of exposure to 1 per cent. phenol.

Using the nephelometer readings in Table III on the graph in Figure 3 the reaction times corresponding to various survivor levels were obtained —the dotted lines in Figure 3 illustrate the method with reference to the 0·5 per cent., 1·0 per cent. and 5·0 per cent. survivor levels. In this way it is possible to draw up, for each experiment, a table (Table V) showing the reaction times corresponding to various survivor levels.

TABLE V

REACTION TIMES CORRESPONDING TO VARIOUS SURVIVOR LEVELS

Survivors	0·5	0·6	0·7	0·8	0·9	1·0	2·0	3·0	4·0	5·0	6·0	7·0	8·0	9·0	10·0
Reaction Times	12·1	11·2	10·7	10·2	9·8	9·5	7·7	6·8	6·2	5·7	5·2	4·9	4·6	4·3	4·1

The principles of this technique were applied to tests on 1·0 per cent. and 0·9 per cent. phenol using 20 drops, 10 drops, and 5 drops samples, and 0·8 per cent. phenol using 5 drops and 10 drops samples ; by analysis of the results it was expected that the effects of the varying size of sample and the difference in strength of the phenol would be evident. The arithmetical averages of the experiments were plotted on Figure 4.

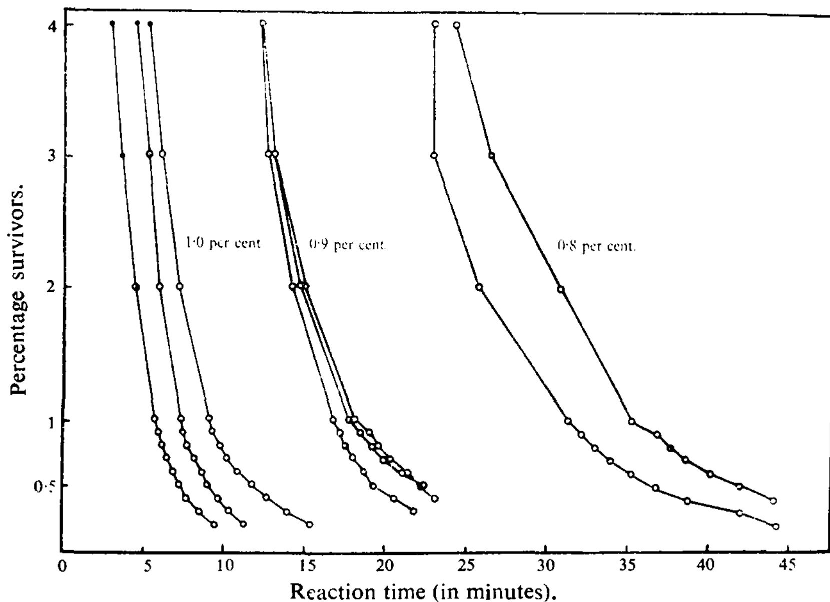

FIG 4. Reaction time—percentage survivor graph for *Bact. coli* against various percentages of phenol.

ANALYSIS OF RESULTS

Linear Regressions. The curves plotted in Figure 4 were of such a shape, as to warrant further investigation.

Curvilinear relationships, such as those of Figure 4 would be extremely cumbersome mathematically and it was desirable if possible to convert the results into linear regressions. Gaddum[3] and Hemmingson[4] suggested that individual sensitivities of experimental animals to many drugs

would exhibit a normal distribution if logarithms of the doses were used to plot the dose effect curve. Withell[5] showed that the resistances of bacteria to bactericides were normally distributed if a logarithmic scale was used to plot the reaction time on a survivor-reaction time regression. Applying a logarithmic scale to the curves of Figure 4 gave a fresh series of curves, the shapes of which more nearly approximated the lower portion of a typical sigmoid curve for an ideal normal distribution. By making use of the normal equivalent deviation (N.E.D.) proposed by Gaddum[3] or the Probit proposed by Bliss[6] such a sigmoid curve is transposed into a straight line. The averages which formed the basis of Figure 4 were then plotted on a probit percentage mortality-log. reaction time graph, and the results showed an approximately linear relationship and thus justified the decision that further analysis of the individual results was desirable.

Taking the results of each experiment the percentages of survivors were converted to percentages of mortality measured in probits and the reaction times to a logarithmic scale. The equation to a linear regression of log. reaction time on probit per cent. mortality was calculated for the results of each experiment. The figures and calculations for one experiment involving 0·8 per cent. phenol with a 5 drops sampling are quoted below and the collected results summarised.

Probit X	X^2	Log. time Y	Y^2	XY
7·6521	58·554634	1·6395	2·687960	12·545618
7·5758	57·392746	1·6233	2·635103	12·297796
7·5121	56·431646	1·6149	2·607902	12·131290
7·4573	55·611323	1·6107	2·594355	12·011473
7·4089	54·891799	1·6064	2·580521	11·901336
7·3656	54·252063	1·6021	2·566436	11·800428
7·3263	53·674672	1·5888	2·524286	11·640025
7·0537	49·754684	1·5211	2·313745	10·729383
6·8808	47·345408	1·4564	2·121101	10·021197
6·7507	45·571950	1·4082	1·983027	9·506336
6·6449	44·154696	1·3655	1·864590	9·073611
6·5548	42·965403	1·3346	1·780890	8·747381
86·1830	620·601024	18·3714	28·259916	132·405674
$\bar{X}=7·1820$		$\bar{Y}=1·5309$		

$$\Sigma(X-\bar{X})^2=1·641900$$
$$\Sigma(Y-\bar{Y})^2=0·134221$$
$$\Sigma(X-\bar{X})(Y-\bar{Y})=0·463811$$

		Sum of Squares	Variance
Total		0·134221	
Due to regression	$\dfrac{[\Sigma(X-\bar{X})(Y-\bar{Y})]^2}{\Sigma(X-\bar{X})^2}$	0·13039	0·13039
Residual		0·00383	0·00038

As the residual variance was small compared with the variance due to

regression it indicated that the data could be expressed in the form of a linear regression.

$$\text{Slope of regression line} \frac{\Sigma(X-\overline{X})(Y-\overline{Y})}{\Sigma(X-\overline{X})^2} = 0{\cdot}2825$$

Hence equation to regression line is :—

from $(y-y^1)=m(x-x^1)$

$$Y=1{\cdot}5309+0{\cdot}2825\ (X-7{\cdot}1820)$$

The summarised results of the equation to the regression lines for other results are given in Table VI.

TABLE VI

SHOWING THE REGRESSION EQUATIONS CALCULATED FROM THE RESULTS OF 40 EXPERIMENTS ON THE BACTERICIDAL VALUE OF PHENOL AGAINST *BACTERIUM COLI*

Percentage Phenol	Number of Drops	Experiment Number	Regression Equation $y=\bar{y}+b(x-\bar{x})$		
			$\bar{y}$	b	$\bar{x}$
0·8	5	105	1·5309	0·3209	7·1820
		106	1·5295	0·3234	7·1820
		107	1·5309	0·2825	7·1820
		108	1·4105	0·4240	7·1820
	10	117	1·4829	0·2786	7·2254
		118	1·5109	0·3165	7·4417
		119	1·4634	0·3297	7·2254
		120	1·3610	0·4637	7·4417
		121	1·5113	0·3124	7·2721
		122	1·5306	0·2485	7·4417
0·9	5	127	1·2550	0·4082	7·1820
		128	1·2172	0·3416	7·1820
		129	1·2109	0·2907	7·2721
		130	1·1971	0·3489	7·2841
		131	1·2515	0·3040	7·1820
		132	1·1871	0·3406	7·3980
	10	123	1·1684	0·2501	7·3272
		124	1·1489	0·1774	7·4417
		125	1·3003	0·3784	7·2813
		126	1·2201	0·2987	7·4417
	20	133	1·2029	0·3867	7·1329
		134	1·1656	0·3952	7·4417
		137	1·2794	0·2879	7·2721
		138	1·2778	0·3007	7·4417
1·0	5	89	1·0043	0·4117	7·3272
		90	0·9727	0·3946	7·3272
		91	0·9884	0·4176	7·3272
		92	0·7887	0·3481	7·3272
	10	111	0·9515	0·3484	7·2721
		112	0·9131	0·4175	7·4417
		113	0·9258	0·3078	7·3272
		114	0·9226	0·2859	7·4417
		115	0·7329	0·3524	7·2721
		116	0·5854	0·2510	7·3272
	20	74	0·7081	0·3766	7·3272
		75	0·4430	0·3570	7·3272
		76	0·9162	0·4314	7·4978
		77	0·7137	0·2339	7·4978
		78	0·8138	0·5619	7·3272
		79	0·8042	0·4141	7·3272
		80	0·7779	0·4126	7·3841

Further analysis of the 0·8 *per cent. Phenol 5-Drop Sample Results.* In this instance there were four regression lines :—

$$\text{(i)}\ Y=1\cdot5309+0\cdot3209\,(X-7\cdot1820)$$
$$\text{(ii)}\ Y=1\cdot5295+0\cdot3234\,(X-7\cdot1820)$$
$$\text{(iii)}\ Y=1\cdot5309+0\cdot2825\,(X-7\cdot1820)$$
$$\text{(iv)}\ Y=1\cdot4105+0\cdot4242\,(X-7\cdot1820)$$

It was desired to find if :—

(a) all the lines could be represented as going through their respective means with the same slope ;

(b) all the lines could be represented by one common line going through the grand mean of Y's (Tippet[7])

(a) *To test if the four lines could be represented as going through their respective means with the same slope.* The residual sums of squares and their corresponding degrees of freedom were :—

	Residual Sum of Squares	Degrees of Freedom
(i)	0·002749	10
(ii)	0·020037	10
(iii)	0·00383	10
(iv)	0·00189	10
	0·028506	40

The sum of these residuals divided by the sum of the degrees of freedom gave an estimate of the residual variance of Y (which was assumed to be constant for all samples).

$$\text{i.e. First estimate of residual variance of Y} \quad \ldots \quad =\frac{0\cdot0285}{40} =0\cdot00071$$

Then, if it was assumed that the samples have one regression coefficient but different means of Y for a given value of X the residual sum of the squares from the regression lines with a common slope and their degrees of freedom were calculated from :—

	$\Sigma(X-\overline{X})$	$\Sigma(Y-\overline{Y})$	$\Sigma(X-\overline{X})(Y-\overline{Y})$	Degrees of Freedom
(i)	1·642	0·171815	0·526969	11
(ii)	1·642	0·191757	0·530987	11
(iii)	1·642	0·134221	0·463811	11
(iv)	1·642	0·297502	0·697110	11
	6·568	0·795595	2·218777	44

from which

$$\text{Residual Sum of Squares}=0\cdot795595-\frac{(2\cdot218777)^2}{6\cdot568} =0\cdot046046$$

the number of degrees of freedom is 43 that is 44 less 1 as one is absorbed in fitting the regression line. Thus :—

$$\text{Second Estimate of Residual Variance of Y}=\frac{0\cdot046046}{43} =0\cdot00107$$

These two estimates of the residual variance in Y (0·00071 and 0·00107) could not be compared as they were not independent. When however the sum of the squares used to get the first estimate (0·028506) was subtracted from the sum of the squares used to get the second estimate (0·046046) and the remainder divided by the difference in the number of degrees of freedom then a third estimate of the variance was obtained which was independent of the first and could be compared with it. This is best summarised :—

Sum of Squares	Degrees of Freedom	Variance
0·046046	43	
0·028506	40	0·00071
0·01754	3	0·00584

from which Variance Ratio (F) $= \frac{0\cdot00584}{0\cdot00071}$

$= 8\cdot22$

(From Tables F at 3 to 40 degrees of freedom is 2·84 at 5 per cent. level.)

Thus, the variances are significantly different and so the four lines (i), (ii), (iii) and (iv) above cannot be regarded as having the same slope.

(b) *To test if the four lines could be represented by one common line going through the grand mean of Y's.*

For this the total regression was calculated as if all the points were scattered along one regression line :—

ΣX	ΣX^2	ΣY	ΣY^2	ΣXY
86·183	620·601024	18·3538	28·263588	132·346449
86·183	620·601024	16·9259	24·171343	122·257513
86·183	620·601024	18·3714	28·259916	132·405674
86·183	620·601024	18·3711	28·296591	132·466578
344·732	2482·404096	72·0222	108·991438	519·476214

From which

$\Sigma(X-\bar{X})^2=6\cdot5676$ $\Sigma(Y-\bar{Y})^2=0\cdot9248$ $\Sigma(X-\bar{X})(Y-\bar{Y})=2\cdot2188$

and

	Sum of Squares	Degrees of Freedom	Variance
Total	0·9248	47	0·0197
Due to regression	0·7496	1	0·7496
Residual	0·1752	46	0·0038

As in case (a) above an independent estimate of variance was calculated and gave :—

	Sum of Squares	Degrees of Freedom	Variance
Residual total	0·1752	46	
Residuals from individual regressions	0·0285	40	0·00071
	0·1467	6	0·02445

from which $$F = \frac{0 \cdot 02445}{0 \cdot 00071} = 34$$

Hence the four lines cannot be regarded as having the same slope and also they lie at different levels.

Later Results. As previously stated above the bottles in the earlier experiments were incubated in an electric incubator whereas for later experiments a constant temperature water-bath was used. Typical results for two experiments using the water-bath are given in Table VII.

TABLE VII

RESULTS OF TWO TYPICAL EXPERIMENTS GIVING THE REACTION TIMES CORRESPONDING TO VARIOUS SURVIVOR LEVELS IN THE ACTION OF 1·0 PER CENT. PHENOL ON A SUSPENSION OF *BACTERIUM COLI*

Percentage Survivors	Reaction Times					
	Experiment A			Experiment B		
0·5	12·1	11·0	12·5	9·7	10·0	11·5
0·6	11·2	9·9	11·4	8·9	9·3	10·8
0·7	10·7	9·3	10·9	8·3	8·8	10·0
0·8	10·2	8·8	10·3	7·9	8·5	9·4
0·9	9·8	8·5	10·0	7·3	8·0	9·0
1·0	9·5	8·2	9·7	7·1	7·7	8·7
2·0	7·7	6·7	8·1	5·1	6·1	7·0
3·0	6·8	6·1	7·2	4·6	5·3	6·0
4·0	6·2	5·7	6·4	4·2	4·8	5·4
5·0	5·7	5·3	5·8	3·9	4·3	5·0
6·0	5·2	5·0	5·4	3·6	4·0	4·6
7·0	4·9	4·8	5·1	3·4	3·7	4·3
8·0	4·6	4·5	4·8	3·3	3·5	4·0
9·0	4·3	4·2	4·4	3·2	3·4	3·8
10·0	4·1	4·0	4·0	3·1	3·2	3·7

The probit mortality-log. reaction time regressions for these were :—

$$Y = 0 \cdot 7534 + 0 \cdot 3865\ (X - 7 \cdot 3272)$$
and
$$Y = 0 \cdot 8201 + 0 \cdot 3784\ (X - 7 \cdot 3272)$$

The sums of the squares and their corresponding degrees of freedom were

	Sum of Squares	Degrees of Freedom
Total (Expt A)	1·433037	44
Due to regression (Expt A)	1·402917	1
Residual (Expt A)	0·030120	43
Total (Expt B)	1·423083	44
Due to regression (Expt B)	1·345134	1
Residual (Expt B)	0·077949	43
Total (A and B)	2·957224	89
Due to regression (A and B)	2·746908	1
Residual (A and B)	0·210316	88

From these results using the analysis outlined above, it can be shown that the two lines could be represented as going through their respective means with the same slope, that is the lines are parallel, but they could not be combined to give a single line representative of 1 per cent. phenol with a 5 drops sampling.

Discussion

The two main criticisms of the second part of this work[1] are partially answered in this third part, the use of a reference turbidity increases the accuracy of measurement, and the results show that more stringent control of incubation temperature considerably reduced the variation in results.

Alper and Sterne[8] and Huntingdon and Winslow[9] have shown that the size of individual bacteria in a *Bact. coli* culture varied considerably during the first 7 hours of growth. It was thus reasonable to assume that two cultures of *Bact. coli* grown from the same size inoculum of the same parent culture would contain the same number of organisms *only* when the conditions of incubation of the two cultures were identical. Such an assumption also precludes the use of a reference turbidity of the barium sulphate type or the calibration of the nephelometer scale in number of organisms. In this part of the work cultures of inocula from definite dilutions of the original suspension of *Bact. coli* and from a test mixture (of original suspension and bactericide) were incubated under exactly similar conditions and the turbidities were compared : on the assumption that ' n ' bacteria in the test mixture would produce the same turbidity as ' n ' bacteria in the original suspension, then an estimate of the percentage of survivors in the test mixture was made. One criticism of this is that although the conditions of time and temperature of incubation were identical for test and reference cultures the bacteria in the test sample had been subject to the action of the bactericide and they may have been so injured that ' n+a ' bacteria had been taken as an inoculum from the test mixture to produce a turbidity equal to that produced by ' n ' bacteria from the original culture unaffected by the bactericide. This does not however detract from the principle of the reference turbidity since in every case the reference turbidity graph (Figure 2) shows the turbidities produced by 0·5 per cent. to 10·0 per cent. of the original suspension under the conditions of the test. It was realised that in calculations what is referred to as 99·5 per cent. mortality may in fact be very slightly less than this ; until some reliable counting method with extremely small limits of error is available for checking these figures it has been assumed that this small error, inherent in this method, can be neglected.

Withell[5], Jordan and Jacobs[10], and Berry and Michaels[11] in similar work have all calculated Probit-Log. Time regressions since in their work the time has been the independent variable, although contrary to normal procedure all the above workers have used the regressions to calculate a value for the independent variable for a known value of the dependent variable, that is the time for a given mortality. In this work however although in the experiments the time was the independent variable in the results obtained for plotting the final graphs the percentage of survivors became the independent variable and thus allowed a regression of log. time on probit per cent. mortality to be calculated. The equation to such a regression line is :

$$Y = \bar{Y} + b(X - \bar{X})$$

where Y=expected value of log. reaction time
X=value of probit per cent. mortality for which Y is desired
$\bar{X}$ & $\bar{Y}$=mean values of X and Y obtained in test
b=regression coefficient (slope of line).

The fact that each experiment consisted of 10 measurements made over the comparatively short range of mortality, namely 90 per cent. to 99·5 per cent., and also the fact that the correlation coefficients were all very close to unity, justified the conclusion that the calculated regression was linear. The correlation coefficients being close to unity (0·972 and 0·989) means also that the regression coefficients calculated (0·387 and 0·378) would approximate the reciprocals for those calculated for probit-log. time regressions ; in this respect it is interesting to note that the reciprocals of the coefficients calculated by Withell[5] for 0·5 per cent. phenol against *Bact. coli* at 20°C. were 0·5, 0·32 and 0·30, and two of these are of the same order as the above coefficients. Withell's coefficients were calculated from data spread over a much wider mortality range, but similarity of the above coefficients and a consideration of the values of the coefficients obtained for 1·0 per cent., 0·9 per cent. and 0·8 per cent. as listed in Table VI are an indication that the probit-log. time regressions for all strengths of phenol against *Bact. coli* will be parallel.

The slopes of the regression lines for 0·8 per cent. phenol in the earlier experiments were shown to be significantly different but, in later experiments in which the temperature of incubation was more constant this difference in slope was reduced and the lines obtained may be reasonably taken as parallel and this permits of a comparison of these two later lines. For a complete comparison adjustment to the common regression of 0·382 would be necessary but, since in both experiments $\bar{X}$ had the same value then the two values of $\bar{Y}$ afford a means of estimating the difference in level of the two lines and this shows a difference of only 0·94 minute in the reaction time at a mortality level of approximately 97·5 per cent. This difference is a measure of the day to day variation and is considerably smaller than the difference in levels obtained by using different strengths of phenol, as expressed by the averages in Table VIII.

TABLE VIII

SHOWING THE DIFFERENCE IN AVERAGE LEVELS OF THE REGRESSION LINES OBTAINED USING DIFFERENT STRENGTHS OF PHENOL AGAINST *BACTERIUM COLI*

Phenol	$\bar{Y}$ at $\bar{X}$=7·3272	Standard Deviation
per cent.		
0·8	1·50	0·07
0·9	1·22	0·06
1·0	0·81	0·12

Thus the daily variation which completely masked the results in Part II[1] has been reduced to a level that permits of closer examination of results.

Berry and Michaels[12] have shown that the concentration of the initial inoculum affects the velocity of the bactericidal action. In this respect

a more rigid control of the initial suspension may help in reducing even further the daily variation.

SUMMARY

1. To reduce the daily variation noted earlier a new form of reference turbidity for use with nephelometers has been introduced. This consists of diluting the initial suspension of *Bact. coli* from 10 per cent. to 0·5 per cent. with sterile water and incubating inocula from these dilutions under conditions identical with those for similar inocula from the bactericidal test on the same initial suspension; comparison of turbidities after 5 hours incubation enables a time survivor relationship to be calculated.

2. The analysis of results obtained by this new method still showed an unduly large variation which was later reduced by more efficient control of incubation temperature and the final results showed that parallel time-survivor regression lines were obtained, although complete reproducibility of results without a small daily variation appears to be more difficult. There are however indications that the technique will allow of comparisons between different strengths of the same bactericide.

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