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

PART II. PRELIMINARY INVESTIGATIONS IN THE USE OF THE NEPHELOMETER

BY H. BERRY AND A. M. COOK

From the Department of Pharmaceutics, School of Pharmacy, University of London

Received January 20, 1950

THERE are many methods available for the evaluation of bactericidal action, and these may be classified as follows. (1) End point methods in which tests for sterility are performed on samples taken after a fixed time or at predetermined time intervals from a test solution of the bactericide which has been inoculated with the test organism. (2) Counting techniques from which a death rate or time-survivor regression can be calculated. (3) Methods involving measurement of the respiration rate or oxygen uptake. (4) Turbidimetric methods which can be divided into two sub-groups: (a) Those measuring the extent of the inhibition of growth of the organisms in presence of the bactericide. (b) Those used for counting the survivors and from which death rate or time-survivor regressions can be calculated. (5) *In vivo* tests.

In all *in vitro* methods the following factors must be taken into consideration. (a) The concentration of the bactericide; (b) The temperature of the reaction; (c) The time of exposure of the bacteria to the bactericide; (d) The medium in which the reaction takes place; (e) The test organism used, and even the strain and its history prior to use; (f) The concentration of organisms used as an inoculum. Variations in one or more of these factors will affect the result, and in any accurate and dependable measurement all but one of these factors must be kept constant.

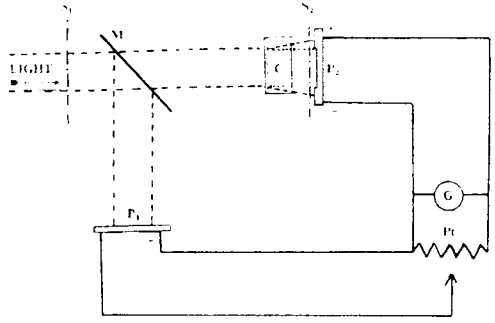
In this work the action of phenol on *Bacterium coli* was studied. The time of exposure of the bacteria to the bactericide was the variable, and a nephelometer was used to count the number of survivors, with the object of calculating a time-survivor regression and examining the reproducibility of such regressions.

EXPERIMENTAL

Nephelometer. The nephelometer used was the compensated photometric type described by Needham[1]. It consisted essentially of a collimated beam of light which was divided by interposing a sheet of optically flat, plain glass at 45°C. in this beam. The portion of the beam reflected from the glass fell on the compensating photoelectric cell (P_1); the other portion of the beam passed through a flow-through type of glass cell having two optically flat sides 3 cm. apart and a drain vent at the base to enable the bacterial suspension to be drawn off; the light

from this cell then fell on the second photocell (P_2). From the centre of the sensitive surface of this second photocell P_2 a disc, slightly larger in diameter than the light beam, had been removed, thus only light scattered by the suspension activated this second photocell. The output from this second photocell was connected across a reflecting galvanometer, whilst that of the first photocell was connected across a potentiometer, graduated 0 – 100, and thence with reversed polarity to the galvanometer.

Figure 1 is a diagrammatic sketch of the nephelometer and circuit.



$P_1 : P_2$ —Photocells. $S_1 : S_2$—Shutters.
G —Galvanometer. C —Cell for suspension.
Pt —Potentiometer. M —Sheet of plain glass.

FIG. 1.—Diagrammatic sketch of nephelometer

The apparatus was also fitted with two shutters S_1 in the main beam and S_2 in the beam incident on the second photocell.

Reference to the circuit diagram shows that the compensating photocell P_1 acts as a reference standard, and before any measurements could be taken this reference had to be set up at an arbitrary value, which, once chosen, had to be maintained throughout the whole work. To do this, the procedure at the beginning of any measurement was as follows:—(a) With both shutters closed the instrument was switched on and allowed 2 minutes for the light circuit to reach equilibrium. (b) A 66 per cent. transmission filter was placed in front of the photocell P_1 and the potentiometer dial was set at 75. (c) The main beam shutter was opened and the intensity of the light adjusted, by means of a variable resistance in the light circuit, until the galvanometer showed a deflection corresponding to 10 μamps. (d) The main shutter was closed and the filter removed.

During the course of an experiment checks were periodically made by isolating the photocell P_2, reinserting the 66 per cent. filter, setting the potentiometer at 75 and noting if the galvanometer reading still corresponded to 10 μamps.

The method of using the nephelometer once it was set up as described was:—(a) The shutter in front of photocell P_2 was opened. (b) The suspension whose turbidity was to be measured was poured into the cell. (c) The main beam shutter was opened—this caused the galvanometer

to swing according to the relative response of the two photosensitive surfaces. (d) The potentiometer was adjusted until the galvanometer showed no deflection, and this reading of the potentiometer was recorded. (e) The main beam shutter was closed and the suspension discarded. (f) The next suspension was run into the cell or, if no more readings were to be taken, the cell was sterilised by a 2 per cent. solution of phenol in alcohol and the instrument switched off.

Standard Capillary Dropping Pipettes. The pipettes were made and cleaned according to the method described by Withell[2] and Berry and Michaels[3], with the exception that an 0·5 per cent. Teepol solution was used in place of the 5 per cent. solution used by Berry and Michaels.

Throughout the experiments the use of tap water for washing glassware was avoided where possible, and, where any glass apparatus had been washed in tap water, at least three separate and distinct rinsings with distilled water always followed. In this way, it was found that the formation of the white scale, often noted on glassware after repeated use, washing and dry heat sterilisation, was considerably reduced. The pipettes, after cleansing in Teepol solution, were rinsed in hot distilled water, and dried in a warm oven. The tip of each pipette was examined, initially and after each experiment, under a X,2. magnifying lens and those with uneven or rough edges were rejected. The wide ends of the satisfactory pipettes were plugged with non-absorbent cotton-wool, packed in glass tubes, and sterilised by dry heat at 150°C. for 1 hour. After use, when the pipettes were contaminated with bacteria, they were placed in a flat enamelled or pyrex dish and autoclaved at 10 lbs. for 1 hour and then the cleansing cycle repeated.

Other Pipettes. The 25 ml., 20 ml., 10 ml., 5 ml., and 2 ml. pipettes used in the experiments were all normal laboratory grade B type; grade A were not used since repeated dry heat sterilisation would reduce the accuracy of such pipettes. When possible, pipettes with reasonably wide bore tips were chosen in order to keep the times for filling and emptying to a minimum. To facilitate the choice of the correct pipette, the wide bore ends of the pipettes were plugged with cotton-wool, a different coloured wool being used for each size of pipette. Sufficient pipettes for one experiment were packed in a copper tube 2 ft. × 3 in. diam. and sterilised by dry heat.

Medication Tubes. These were standard 50 ml. Pyrex glass boiling tubes with standard (B19 or B24) ground-glass stoppers.

Culture Bottles. 4-oz. flat-sided glass bottles closed by aluminium screw caps and fitted with rubber washers were found to be the most convenient for culture bottles.

An experiment was devised to compare the suitability of the glass-stoppered boiling tube (used as medicating-tubes) with that of 4-oz. screw-capped bottles as culture containers.

The original intention was to use 50 ml. of peptone broth for culture purposes, but as 50 ml. completely filled most of the glass-stoppered tubes, for the purpose of this experiment only 45 ml. of broth was used in each case.

(a) Experiment:—(i) 12 bottles and 12 tubes, each containing 45 ml. of sterile peptone broth, were placed in an incubator at 37°C. overnight. They were then divided into three groups, each group containing 4 bottles and 4 tubes.

(ii) Into the bottles and tubes of the first group were placed 5 drops of a 24-hour culture of *Bact. coli*, into those of the second group 10 drops of the same culture and into that of the third group 15 drops.

(iii) One tube and one bottle from each group were, respectively, incubated for 2, 3, 4 and 5 hours, and the turbidities then measured on the nephelometer.

(b) Results:—The results, shown graphically in Figure 2, illustrate that a difference does exist between bottles and tubes.

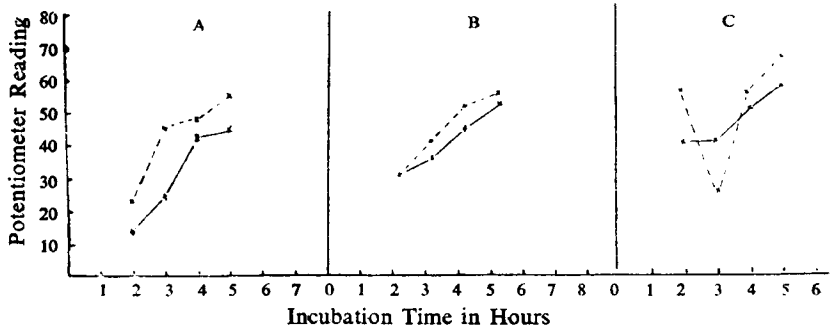


FIG. 2.—Showing comparison of turbidity produced when 5 drops (A), 10 drops (B) and 15 drops (C) of a 24-hr. culture of *Bact. coli* were incubated in bottles (broken lines) and tubes (continuous lines)

(c) Discussion:—The difference between bottles and tubes may be attributed to the greater volume of air above the culture medium in the bottles when compared with tubes, which permits of more rapid aerobic growth. The results clearly indicated the necessity for a standardised culture bottle. A sufficient quantity of 4-oz. bottles for the complete work was obtained. *p*H tests on distilled water autoclaved in these bottles indicated that considerable amounts of alkali were leached out of the glass and so the whole consignment of bottles was autoclaved with acid, followed by washing and double autoclaving with distilled water until no measurable amount of leaching was observed.

Withell[2] pointed out that there was reason to believe that freshly vulcanised rubber liberated substances which are bactericidal to some organisms and, to counteract this, all new rubber discs used in the culture bottles were autoclaved before use. The absorption by rubber caps, of phenol-like substances, which occurs on autoclaving was not a factor in the present series of experiments as the culture bottles were never autoclaved whilst containing any phenolic substances.

Culture Tubes. The test tubes employed for agar slopes were of pyrex glass 6 in. by $\frac{5}{8}$ in.

Materials. Liquid Medium. The importance of a uniform culture medium in bactericidal evaluation was stressed by Wright[4], who found

that different samples of peptone gave different phenol coefficients; this variation in peptones was also noted by Philbrick[5], and Brewer[6] suggested that the variation may be due to the effect of added substances on the phospholipide content of the peptone.

Needham[7] in his original paper on the nephelometer suggested the simple formula:—

"Oxoid" peptone	10 g.
Sodium Chloride (A.R.)	5 g.
Distilled water	1000 ml.

In his experiments with peptones, "Oxoid" peptone not only proved superior to five other varieties of peptone, but he also showed that, with the use of "Oxoid" peptone, very little advantage was derived from the addition of the customary meat extract. Experiments also showed that only slightly heavier growth resulted from the use of 2 per cent. peptone. The substitution of the mixture of salts employed in Ringer's solution for the sodium chloride in the suggested formula resulted in a precipitate on heating and necessitated a further filtration, and so was not substituted in the formula. The simplicity and ease of preparation of such a culture medium commended it, and it was used throughout these experiments. To eliminate any inter-batch variation in the peptone, as noted by Brewer, a large consignment of one batch of "Oxoid" peptone was kept exclusively for this work.

The method of preparation was to dissolve the solids in water (no heat is required if only 1 per cent. peptone is used); the *p*H was adjusted to 7·3 by the addition of N potassium hydroxide. A careful check was kept on the amount of potassium hydroxide solution added, and on no occasion did the solution require N potassium hydroxide outside the limits of 4·0 $\pm$ 0·5 ml. of medium. The broth was filtered and distributed by an Ayling filter into 4-oz. culture bottles. The broth was then sterilised by autoclaving at 10 lbs. for 20 minutes. On occasions, for easier storage purposes 5 litres of double-strength broth were made and diluted with freshly boiled and cooled distilled water and refiltered before distribution and autoclaving.

A routine check of *p*H after autoclaving was carried out on a sample bottle from each batch of medium made. A regular drop of 0·1 units of *p*H was observed in almost every case.

Solid Media. 2 per cent. of powdered agar was added to some of the peptone water prepared as above, and warmed until the agar dissolved. The molten material was clarified by passing through washed paper pulp and distributed into tubes which were plunged and sterilised by autoclaving for 30 minutes at 10 lbs. Before the molten material had set, the tubes were removed from the autoclave and laid in a sloping position until the medium solidified. The tubes were stored in a dust-proof tin at room temperature.

Test Organism. 24 hour cultures of *Bact. coli* (Lister Institute No. 5933) were freeze-dried and the material distributed into a number of sterile tubes and sealed. These cultures were stored at room temperature.

At the beginning of each month a tube of freeze-dried material was opened and the contents suspended in a little sterile distilled water. Four agar slopes were inoculated with this suspension and incubated at 37°C. for 24 hours. These cultures were classed as master slopes, sealed with paraffin wax and stored at room temperature; no master slope was used if it had been stored for longer than 2 months.

A portion of the growth of one of the master slopes was removed with a sterile platinum loop and used to inoculate four fresh slopes; after 24-hours incubation at 37°C. these slopes were taken from the incubator and a small portion of the growth of each slope was used to sub-culture on 4 fresh slopes. This 24-hour subculturing was performed daily, and only slopes from the 4th to 14th subculture were used in the experiments.

Distilled Water. The water used throughout this work was distilled from a " Baracop " still, which, fitted with an efficient baffle to prevent entrainment, and a glass condenser, supplied a distilled water free from the metallic impurities noted by Davis[8] and Wilson[9,10].

Phenol. Standard 5 per cent. solutions in freshly boiled and cooled distilled water were made up each week from analytical grade phenol. Solutions of greater dilution were made up from these standard solutions just prior to each experiment.

Preliminary Investigations

Turbidity Response Curve. In his review of photoelectric instruments, Baier[11] points out that although nephelometers are sensitive to small amounts of turbidity the linearity between the intensity of light measured and the amount of suspended matter holds moderately well within narrow limits when the sample is faintly turbid, but with increasing turbidity the effect of secondary absorption may become so great as to give a lowering of the intensity of the scattered light. The following experiment was performed to examine the linearity of the dose response curve of the nephelometer.

Fourteen bottles, each containing 50 ml. of sterile peptone broth were taken; into these bottles, from the same sterile standard dropping pipette, were dropped 1, 2, 5, 10, 15, 20, 25, 30, 40, 50, 75, 90, and 110 drops respectively of a 24-hour culture of *Bact. coli.* Immediately the bottles were shaken and the turbidity measured on the nephelometer. The results were plotted on Figure 3.

These results showed that, assuming each drop of the 24-hour culture contained approximately the same number of bacteria, a linear relationship exists between nephelometer reading and number of bacteria for readings up to 80. The values of the nephelometer reading above 80 were below those anticipated by the straight line, dotted in Figure 3, and indicated that a little secondary absorption might be taking place.

Effects of Size of Sample and Period of Incubation. To measure these effects 35 culture bottles, each containing 50 ml. of sterile peptone broth were stored in an incubator until a constant temperature of 37°C. had been attained. They were then divided into 5 groups with 7 bottles in

each group; each group subsequently received the same treatment. Nil, 1 drop, 2, 4, 8, 16, and 32 drops, respectively, of a 24-hour culture of *Bact. coli* were dropped, from a standard dropping pipette, into the 7 bottles of each group. The groups were then incubated for 2, 3, 4, 5 and

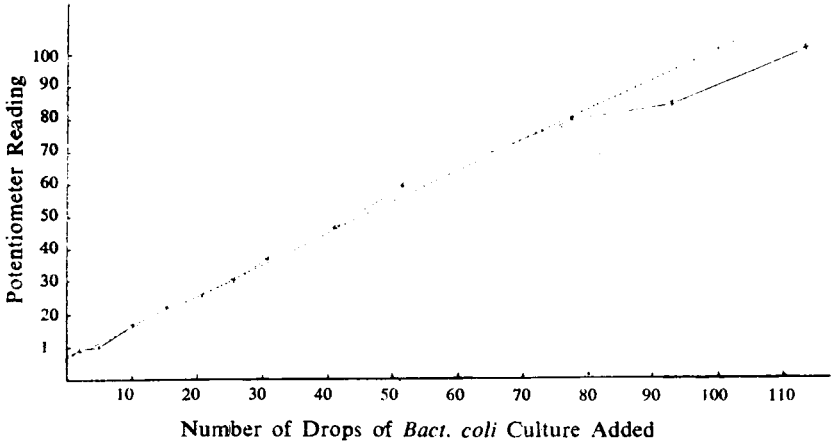


FIG. 3.—Turbidity Response Curve. Showing the turbidity produced when drops of a 24-hr. culture of *Bact. coli* were added to 50 ml. of peptone broth.

6 hours respectively at 37°C. At the end of the incubation period the turbidities of the suspensions were read on the nephelometer. Table 1 shows the results obtained.

It was anticipated that a time survivor curve for something less than 20 per cent. of survivors would be plotted, and the results of Table 1 would be for 100 per cent. of survivors.

TABLE I

TURBIDITIES (QUOTED AS POTENTIOMETER READINGS) PRODUCED WHEN VARIOUS NUMBERS OF DROPS OF A 24-HOUR CULTURE OF *Bact. coli* WERE INCUBATED, IN 50 ML. OF BROTH, FOR VARYING PERIODS

No. of Drops	Turbidities corresponding to incubation periods				
	2 hr.	3 hr.	4 hr.	5 hr.	6 hr.
0	5	6	8	7	6
1	19	18	58	87	98
2	9	31	78	95	97
4	13	51	87	89	B.S.
8	23	71	93	98	B.S.
16	46	85	95	B.S.	B.S.
32	71	94	97	B.S.	B.S.

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

Wilson[9] established the fact, that, in using the standard dropping pipette for bacterial suspension measurement it was inadvisable to use less than 4 drops. Also, Alper and Sterne[12] found that the optical behaviour of the individual cells of a growing culture did not approach that of the parent culture until the culture was 6 to 7 hours old, and Huntingdon and Winslow[13] showed that the cell volume of individual cells in such a

culture of *Bact. coli* was the same as that of the parent culture after 5½ hours' growth.

Thus the minimal time for incubation would appear to be 5 to 7 hours. Considering initial samples of 20, 10 or 5 drops then 20 per cent. survivors from these samples would correspond to 4, 2, or 1 drops respectively in Table 1. From a consideration of Table 1 for a 5-hour incubation period, a maximum survivor level of 5 per cent. could be measured for a 20-drop sample and 20 per cent. survivors for a 5-drop sample. For incubation periods of more than 5 hours only much smaller percentages of survivors would be readable.

Use of a Suspension of Bacteria. The variations in the opacity of a 24-hour culture of *Bact. coli* was unknown, and the opacity was too great for direct measurement on the nephelometer. A sample of such a culture could have been diluted and then measured on the nephelometer, and hence would have acted as a measure of the opacity of the original culture. This sampling would be necessary because the chances of contamination during a nephelometer reading were high and hence any sample used in the nephelometer must be discarded after measurement. With such a method of standardisation any 24-hour culture which showed more than standard opacity would have to be diluted with fresh sterile peptone broth until its opacity equalled that of a standard; and, as no method was available for increasing the opacity of any culture, then a standard would have to be chosen such that its opacity would be below that of any 24-hour culture; and each 24-hour culture would have to be diluted, maybe with considerable amounts of sterile peptone broth. The introduction of fresh sterile medium would result in renewed growth of the culture at any time before and perhaps also during the disinfecting period.

The idea of using a standardised 24-hour culture was discarded in favour of a standard suspension in a vehicle which did not encourage growth subsequent to standardisation.

Bean and Berry[14] have shown that Wilson's modification of Ringer's solution, the suitability of which in the majority of cases was proved by Berry and Michaels[3], should not be used in presence of soap solutions. Since a test of solutions of chloroxylenol in potassium ricinoleate was envisaged, Ringer's solution could not be used for the suspension and so distilled water was used.

Preparation of Suspension. The organisms from 3 of the current agar slope subcultures were washed off into a culture bottle containing 50 ml. of sterile water and a few glass beads. This suspension was shaken for about 1 minute to break up any clumps of organisms. Half (25 ml.) of this suspension was withdrawn and transferred to an empty sterile culture bottle, diluted with 25 ml. of distilled water and used in the nephelometer. The nephelometer was set up with a 50 per cent. transmission filter in the main light beam. The freshly prepared and diluted suspension was poured into the optical cell and both photocells switched in. The potentiometer setting for a zero galvanometer reading was noted. By a system of trial and error sufficient distilled water was measured into the

optical cell until the galvanometer gave a zero reading with the potentiometer set at 50. The amount of distilled water necessary was recorded and the suspension then discarded. From the amount of distilled water added to half the original suspension it was possible to calculate the amount of sterile water to be added to the remaining 25 ml. of suspension such that the suspension formed, if diluted with an equal quantity of water, would give a reading of zero on the galvanometer, with the potentiometer set at 50 if placed in the nephelometer with a 50 per cent. transmission filter in the main beam. Checks with a Burroughs Wellcome Opacity Set and a plate count indicated that such a suspension contained approximately 175 million organisms per ml. of suspension.

Comparison of Suspension and 24-hour Culture. To compare such a suspension with a 24-hour culture the following experiment was performed: Six culture bottles each containing 50 ml. of sterile peptone broth at 37°C. were taken. 1, 2, 4, 8, 16 and 32 drops of a trial suspension, respectively were dropped from a standard dropping pipette into the bottles and they were then incubated for 3 hours and their turbidities measured.

The experiment was repeated using a 24-hour culture in place of the suspension. The results are tabulated in Table II.

These results show that a suspension of *Bact. coli* of the stated opacity approximates in activity a 24-hour culture of the organism.

Reproducibility of Suspension. Further 3-hour incubation tests were performed on suspensions made for other experiments to investigate the

TABLE II

SHOWING THE TURBIDITIES PRODUCED AFTER 3-HOURS INCUBATION OF VARIOUS NUMBERS OF DROPS OF A SUSPENSION, AND 24-HOUR CULTURE OF *BACT. COLI*.

	Turbidity (measured as Nephelometer Readings)					
No. of drops ...	1	2	4	8	16	32
Suspension	15	45	47	84	89	96
24-hour culture ...	18	31	51	71	85	94

TABLE III

SHOWING THE TURBIDITIES PRODUCED AFTER 3-HOURS INCUBATION OF VARIOUS NUMBERS OF DROPS OF SEVERAL SUSPENSIONS OF *BACT. COLI*.

	Turbidity (measured as Nephelometer Readings)					
No. of drops ...	0	1	2	5	10	20
Experiment No.						
34	6	22	37	66	81	98
35	3	19	47	72	91	B.S.
36	4	27	44	65	80	92
37	5	20	32	54	75	90
38	6	26	45	65	80	97
39	12	29	54	76	88	B.S.
40	6	30	63	78	92	97
41	4	20	40	71	84	97
42	4	32	58	75	91	97

reproducibility of the suspension. Table III shows the results of these tests.

These results, whilst showing a fair measure of agreement, were not analysed, since the temperature during the 3-hour incubation period was not constant.

Bactericidal Value of Phenol

Reaction Time. The time during which the bacteria are in actual contact with the bactericide is called, throughout this work " reaction time."

Preliminary Experiments. Preliminary experiments had shown that with 0·75 per cent. phenol, reaction times of 30 to 50 minutes would produce turbidities within the nephelometer range.

Technique. Concentrations of phenol of 0·72 per cent., 0·74 per cent., 0·76 per cent. and 0·78 per cent. were chosen as the strengths for use in these investigations.

(1) 50 bottles each containing 50 ml. of sterile peptone broth were stored in an incubator at 37°C. until just prior to the experiment, when they were transferred to a water-bath at 37°C.

(2) A standard suspension of *Bact. coli* was prepared and placed in a water-bath at 20°C.

(3) Solutions of phenol of 3/2 times the test strength were prepared from a standard 5 per cent. solution by dilution with freshly boiled and cooled distilled water. When prepared, these solutions were placed in the water-bath at 20°C.

(4) 4 Sterile glass-stoppered medication tubes were placed in the water-bath. A sufficient length of time was allowed for all temperatures to reach a constant level.

(5) 10 ml. of the first phenol dilution was transferred by means of a sterile pipette to one of the medication tubes and then the tube returned to the 20°C. water-bath.

(6) At a time zero, 5 ml. of the standard suspension was added to the dilute phenol in the medication tube which was shaken and returned to the water bath.

(7) At a time (zero + t − ½) the medication tube was taken from the water-bath, shaken, and a sterile dropping pipette filled with the phenol-suspension mixture and the medication tube returned to the water-bath.

(8) At a time (zero + t) 20 drops were dropped from the pipette into one of the culture bottles containing the 50 ml. of sterile peptone broth, which was then returned to the water-bath at 37°C. The addition of drops was started 10 seconds before (zero + t) minutes, and by adding one drop per second, the mean time for the addition of the 20 drops was (zero + t.).

(9) Further samples of 20 drops of the reaction mixture were transferred to culture bottles at zero + t + 1, + 2, + 3, ... + 10 minutes respectively.

Operations (2) and (5)-(9) were conducted under aseptic conditions.

(10) The operations (5)-(9) were repeated for the other dilutions of phenol.

(11) The turbidities of the suspensions were read on the nephelometer after exactly 5 hours' incubation.

Results. The results of two consecutive experiments are shown in Table IV.

TABLE IV

SHOWING THE TURBIDITIES PRODUCED APTER 5 HOURS INCUBATION OF 20 DROPS OF REACTION MIXTURES CONTAINING 0.72 PER CENT., 0.74 PER CENT., 0.76 PER CENT., AND 0.78 PER CENT. PHENOL.

Turbidity (measured in Nephelometer Readings)

0·72 per cent. Phenol.

Reaction Time, minutes ...	50	51	52	53	54	55	56	57	58	59
Expt. No. 38	84	92	89	90	87	78	63	61	66	65
Expt. No. 39	82	83	98	79	77	70	68	70	73	70

0·74 per cent. Phenol.

Reaction Time, minutes ...	40	41	42	43	44	45	46	47	48	49
Expt. No. 38	75	77	60	59	62	45	35	27	23	29
Expt. No. 39	66	64	62	62	61	58	39	40	37	30

0·76 per cent. Phenol.

Reaction Time, minutes ...	30	31	32	33	34	35	36	37	38	39
Expt. No. 38	70	64	56	60	57	49	33	29	30	26
Expt. No. 39	93	90	85	86	76	74	73	66	63	64

0·78 per cent. Phenol.

Reaction Time, minutes ...	20	21	22	23	24	25	26	27	28	29
Expt. No. 38	81	73	65	69	64	65	46	45	40	41
Expt. No. 39	B.S.	B.S.	99	98	96	93	89	83	80	78

Extrapolating in the case of 0·78 per cent. the turbidities at 30 minutes would be 37 in Experiment No. 38 and 75 in Experiment 39.

Thus we have for 30 minutes.

0·78 per cent. phenol in Expt. 38 :— 37

Average 66

0·78 per cent. phenol in Expt. 39 :— 75

0·76 per cent. phenol in Expt. 38 :— 70

Average 82

0·76 per cent. phenol in Expt. 39 :— 93

Thus the difference due to change of phenol strength is 16 and the difference due to daily variation (value in Expt. 39 – corresponding value in Expt. 38) is 38 for 0·78 per cent. phenol and 23 for 0·76 per cent. phenol. In this instance, the daily variation was greater than that due to phenol strengths; if then the experiments are to be used for differentiating between the different strengths of bactericides, this daily variation would have to be reduced. To eliminate the effect of the daily variation, the results for 28 experiments were averaged; these results, plotted in

Figure 4, showed a definite difference between the various strengths of phenol used, but insufficient similarity between the graphs to warrant a detailed analysis.

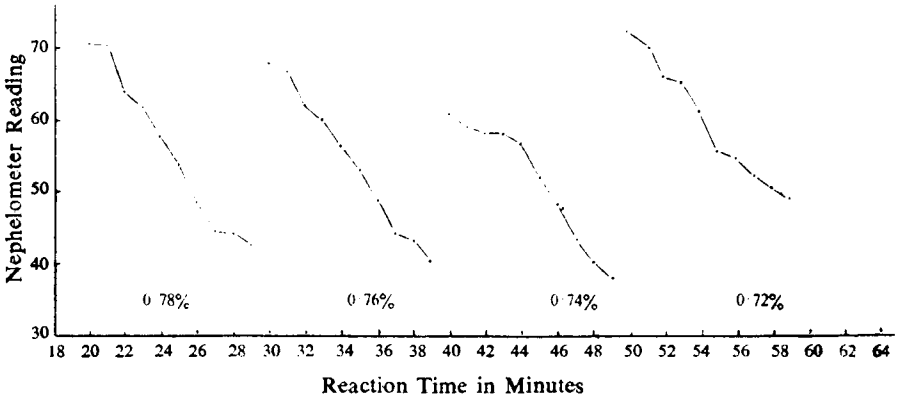


FIG. 4.—Showing the average turbidities produced using 4 different strengths of phenol for various reaction times.

DISCUSSION

Baier[7] has pointed out that with photoelectric nephelometers the linearity between the amount of suspended matter and nephelometer reading holds only for comparatively small amounts of suspended matter. The turbidity response curve plotted in Figure 3 shows that used under similar conditions linearity does hold for the nephelometer, although readings over 90 on the nephelometer should be treated with caution. Considering the findings of Alper and Sterne[8] and Huntingdon and Winslow[9] in connection with the changes in cell size and opacity of young cultures of *Bact. coli*, and having regard to the length of a normal working day and the result shown in Table I the optimal time in incubation appears to be 5 hours with the use of samples of a size less than 20 drops.

The reasons for use of a standard suspension of bacteria instead of a 24-hour culture have already been outlined.

Phenol was used as the bactericide in the preliminary tests because the day to day variations in phenol solutions would be very small and so permit of study of the variations due to experimental technique, and not in order that at a later date similar experiments could be performed with other bactericides and a form of " phenol coefficient " calculated.

The main conclusion to be drawn from the results of the experiments on the bactericidal value of phenol was that a large day to day variation was present. The results of the averages for 28 experiments showed that the method could be applied if some reduction of the daily variation could be accomplished. Table III shows that a small variation in the turbidities produced from the standard suspension does exist but this is not large enough to account for the variation in the final results. The

conclusion could then be drawn that the high daily variation was due, not to the variation in preparing the standard but to the treatment of the standard after preparation. This treatment could be divided into three parts: (i) reaction stage, (ii) incubation stage, (iii) measurement. Stage (i) was similar to the reaction stage in other methods of bactericide evaluation and it seemed doubtful if any further elaboration of this stage would reduce the variation. Thus further work on this method was concentrated on stages (ii) and (iii).

SUMMARY

1. Samples were taken, at predetermined time intervals, from a reaction mixture of phenol and *Bact. coli*, and inoculated into sterile peptone broth. The turbidity of these samples after incubation was measured with a nephelometer in an effort to plot a time-survivor curve.

2. The turbidity-response curve of the nephelometer was found to be a straight line. A 5 to 20 drop sample and a 5 hour incubation period proved to be the most advantageous.

3. The daily variation in the results showed that better control of incubation and turbidity measurement was needed before reproducible results could be anticipated.

REFERENCES

1. Needham, *Nature*, 1946, **157**, 374.
2. Withell, *Quart. J. Pharm. Pharmacol.*, 1938, **11**, 736.
3. Berry and Michaels, *Quart. J. Pharm. Pharmacol.*, 1947, **20**, 331.
4. Wright, *J. Bact.*, 1917, **2**, 315.
5. Philbrick, *Soap*, 1937, **13**, (2), 103.
6. Brewer, *Soap*, 1943, **19**, (4), 101.
7. Needham, *J. Hyg., Camb.*, 1947, **45**, 1.
8. Davis, *Quart. J. Pharm. Pharmacol.*, 1940, **8**, 14. 32.
9. Wilson, *J. Bact.*, 1922, **7**, 405.
10. Wilson, *Spec. Rep. Ser. Med. Res. Coun., London*, 1935. No. 206, 38.
11. Baier, *Physiol. Zool.*, 1947, **20**, 172.
12. Alper and Sterne, *J. Hyg., Camb.*, 1938, **33**, 497.
13. Huntingdon and Winslow, *J. Bact.*, 1937, **33**, 123.
14. Berry and Bean, 1949, *private communication.*