STUDIES IN THE DYNAMICS OF DISINFECTION

I. NEW DATA ON THE REACTION BETWEEN PHENOL AND *BACT. COLI* USING AN IMPROVED TECHNIQUE, TOGETHER WITH AN ANALYSIS OF THE DISTRIBUTION OF RESISTANCE AMONGST THE CELLS OF THE BACTERIAL POPULATIONS STUDIED

By R. C. JORDAN, Ph.D. and S. E. JACOBS, Ph.D., *from the Physiology Department, University College of South Wales and Monmouthshire, Cardiff, and the Bacteriological Laboratory, Imperial College of Science and Technology, London*

(With 10 Figures in the Text)

INTRODUCTION

There is a growing awareness of the fact that a full investigation of the physico-chemical laws underlying the mechanism of disinfection has been too long neglected. Recent publications by Garrod (1940) and Albert (1942), amongst others, have stressed the need for more reliable experimental data on the germicidal action of drugs before the nature of the fundamental laws governing disinfection can be more fully elucidated.

In the investigation of this problem studies of the quantitative aspects must take an important part and, indeed, there are many published records of such experiments, but an examination of them shows that the usual technique employed has been far from satisfactory. In general, bacteria grown at a higher temperature in a rich nutrient medium have been starved of food, through dilution of the culture, and chilled, often suddenly, in addition to being exposed to a germicide, so that the observed death-rates might well have been the resultants of three adverse influences and not the product of the germicide alone. Naturally, in short experiments the mortality due to starvation and chilling could be only slight, yet it is conceivable that the alteration in the environment of the bacteria may have had an effect on their resistance to germicides which would be reflected in the observed mortality curves. The importance of standardizing the bacterial culture as far as age, medium and temperature of growth are concerned is great, and this fact has generally been recognized. Also, experiments should not be too brief or changes in the death-rate may be missed. This obviously implies that the concentration of germicide should not be too high and that adequate numbers of bacteria should be used. The tendency in the past has, on the whole, been to employ far too few organisms and too concentrated germicides. These considerations lead to the conclusion that, in order to comply with all these requirements, the germicide should be added directly to a mature culture grown under carefully controlled conditions which are maintained constant throughout the experiment. This method does, indeed, introduce a complication of its own, since some germicides will react with the material in the nutrient medium, thus becoming reduced in effective concentration. However, there are others which can be used in these circumstances.

When, in the course of other work (shortly to be published) on the growth of bacterial populations maintained on a constant food supply, it was found that a ready means was available for obtaining a completely reproducible culture of *Bact. coli* under rigidly controlled conditions, it was decided to employ this as a uniform substrate on which to investigate germicidal action. It had been found that, if a nutrient solution is continuously added at a constant rate to a bacterial inoculum suspended in a large volume of *p*H 7 phosphate buffer solution at 35° C., after an initial increase the viable population remains constant for a period of several weeks. The initial period of increasing viable population can be reduced to a few hours by adding to the buffer solution before inoculation a predetermined quantity of nutrient material, when the viable population rapidly rises to its steady level, at which it is maintained constant by the continuous food addition. There is thus obtained a bacterial culture at a constant temperature and reaction whose viable population is known to remain constant until influenced only by the germicidal agency under investigation.

As a preliminary to a systematic study of some aspects of the intimate mechanism of disinfection, it was decided to study in detail afresh the action of phenol on *Bact. coli*, to which much of the earlier published work had been devoted. The times of reaction between bacterium and germicide were, however, to be extended by using more dilute solutions of phenol than had previously been the case.

During the investigation it became apparent that the technique gave an opportunity to estimate the distribution of resistance of *Bact. coli* to lethal agencies, a point which has been the subject of considerable controversy in the past. Whereas in multicellular organisms it has been possible to demonstrate a normal distribution of resistance to drugs, the data obtained for unicellular organisms have been more conflicting. It is hoped to pursue the investigation of this fundamental fact in bactericidal action further in subsequent work.

EXPERIMENTAL

The automatic syringe mechanism used for the continuous addition of the nutrient solution to the bacterial culture is described in detail elsewhere (Sims & Jordan, 1942). It consists essentially of a 1 ml. Pyrex 'tuberculin' syringe whose plunger is operated automatically at predetermined intervals by a compact series of levers whose function is to convert the rotatory motion of a synchronous motor into a to-and-fro linear movement. By the action of a pair of mercury-weighted glass valves, a known volume of sterile nutrient solution is transferred from a calibrated burette to the culture flask at each complete operation of the syringe. The interval between successive operations of the syringe plunger

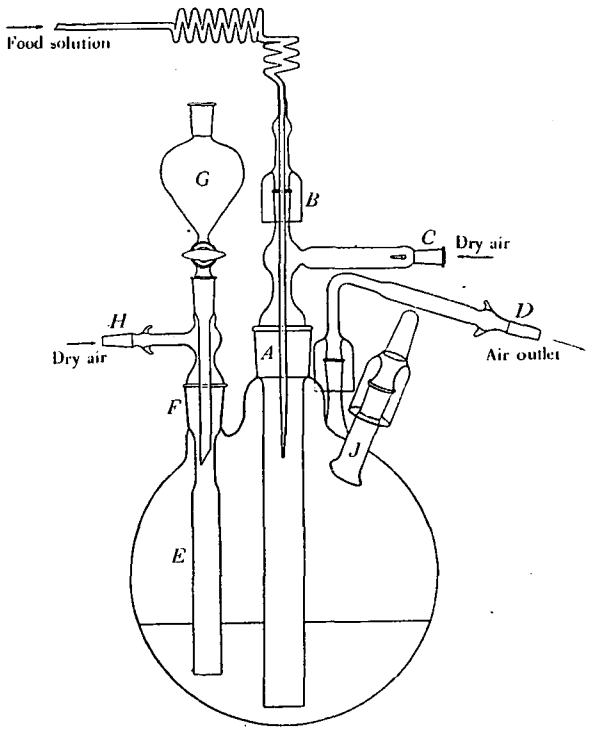

Fig. 1. Diagram of culture flask (see text).

and the extent of its excursion at each operation are capable of wide and rapid variation. The food solution comes into contact with no surface other than glass, and all the parts of the apparatus which require it are capable of steam sterilization and aseptic assembly.

The culture flask used is illustrated in Fig. 1. It consists of a 5 l. Pyrex round-bottom flask with a ground joint *A* on a central wide neck around which are arranged three additional necks, two vertical and one normal to the surface, each with a ground joint. The joint in the central wide neck is sealed by a glass tube, of approximately 28 mm. diameter, which extends to within 1 cm. of the bottom of the flask and which has a narrow ground joint *B* at its upper end, and another, *C*, on a horizontal side tube. Through the dust-proof glass joint *B* passes the flexible glass jet by which the nutrient solution is added to the culture, and a stream of dry, sterile air from Dinton aerators enters through the side tube *C* (which can be plugged with cotton-wool if necessary), bubbles through the culture and escapes from the flask by the neck *D*. By thus surrounding the food jet with a glass guard tube and airstream, contamination of the ingoing broth, by bactéria-laden spray from bursting froth bubbles, is completely prevented and the success of the technique obviously is largely dependent on this fact.

The air, before entering the culture, is dried by sulphuric acid and sterilized by passage through numerous cotton-wool plugs before being warmed in glass coils immersed in the water-bath beneath the culture flask. The functions of the airstream are to stir the culture efficiently, rapidly to distribute the added nutrient solution throughout the culture, to maintain gaseous equilibrium and to evaporate water from the culture at the same rate as the broth is added, thus preserving a constant volume.

The neck *F* carries a second guard tube *E* through which the germicide solution is added from the funnel *G*. A side tube *H* admits a stream of dry, sterile air during the addition in order to distribute the germicide rapidly throughout the entire culture. The required amount of phenol is added as a 5 % solution and, in order to avoid any chance of a high concentration persisting locally for an appreciable time, the solution is added at a slow steady rate over a period of 5 min. By replacing the phenol solution by a dye, it has been shown that dispersion of the added material by the bubbling air is remarkably effective.

Samples are removed from the culture periodically with a sterile pipette via the neck *J*, which is attached normally to the surface of the flask to enable samples to be taken from the centre of the culture.

The culture flask is immersed in a thermostatically controlled water-bath at 35° C. $\pm$ 0·1° up to the unions of the side tubes with the body of the flask and maintained in position by a special clamping device fastened by a supporting framework fixed to the inside of the bath.

Bact. coli, forming as it does a uniform suspension throughout the medium, is a very suitable organism for an investigation of this nature. The culture used is a Type I *Bact. coli*.

The standard technique employed for each experiment is to place in the culture flask 250 ml. stock *p*H 7·0 buffer solution (27·2 g. KH_2PO_4 Analar + 4·55 g. NaOH Analar per l.) and 1300 ml. distilled water containing 1·0 g. Difco dehydrated nutrient broth. With suitable precautions, the flask containing this mixture is then sterilized in steam at 20 lb./sq. in. pressure for 45 min. During autoclaving the volume becomes reduced to about 1450 ml. The food jet, guard tubes and funnel are all wrapped separately in paper and, together with the syringe mechanism, sterilized in steam at 20 lb./sq. in. for 20 min. and then dried at 100° C. for 30 min. in a suitable autoclave. It is advisable to lubricate slightly the outer end of the syringe plunger with a drop of sterile liquid paraffin-vaseline mixture after autoclaving.

The nutrient solution, containing 6 g. Difco dehydrated broth per l., is made up in the 3 l. Pyrex reservoir

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flask (see Sims & Jordan, 1942) and sterilized in steam for 45 min. at 20 lb./sq. in. pressure.

When cool, the culture flask is placed in position in the water-bath, the various parts of the apparatus fitted together aseptically, the airstream and the food supply commenced and the solution in the culture flask inoculated. No trouble has been experienced from contamination of the culture by air-borne organisms either during assembly of the apparatus or during the experiments. All the ground-glass joints in the flask are either covered by glass dust shields or are filled with a film of sterile liquid paraffin.

The standard inoculum is made up as follows. A little growth of *Bact. coli* from a stock agar slant is transferred to 150 ml. broth (8 g. Difco dehydrated broth per l.) and incubated at 35° C. for 24 hr. From this culture 1 ml. is diluted in sterile water to a population of approximately 1×10^5 organisms per ml., and of this suspension 5 ml. are used as inoculum into the 1450 ml. of sterile dilute buffer solution in the experimental culture flask, giving an initial population of 300–400 cells per ml.

As required, a 2 ml. sample of the culture, removed through the side neck *J* and diluted as necessary, according to the probable viable population, with sterile buffer solution of the same molar strength as that used for the culture, is plated out in the usual way except that the modification is made of placing a thin layer of nutrient agar in each Petri dish and allowing it to set before plating out the diluted culture. This procedure is found to give more evenly spaced colonies with a minimum of spreading ones. As many replicate plates are made from each sample as time allows. This is usually six or eight and these are incubated at 35° C. for 48 hr. before the colonies are counted, using the automatic counting device of Sims & Jordan (1941).

Within a few hours of the inoculation the viable population of the experimental culture has risen to about 330 million organisms per ml., at which level it is maintained constant by the continuous addition of broth at the standard rate of 15·2 mg. Difco dehydrated broth per hour. Under these conditions the total, as distinct from the viable, bacterial population slowly rises, but it would appear probable that immediately on the addition of the germicide, even in the lowest concentrations used, active division of the cells ceases although actual mortality may not set in at once.

When the experiment has been in progress for about 40 hr. from the inoculation, the phenol solution is run in and the period of germicidal action is deemed to have begun from the end of the 5 min. taken for the addition. All volumes thereafter removed from the culture are recorded, and at the conclusion of each experiment the volume of culture remaining is determined. From these records and the known volume of germicide solution added, the concentration of phenol is accurately calculated.

Before attempting to interpret the results, the reliability of the bacterial plate counts must be examined. Fisher, Thornton & Mackenzie (1922) have shown that under ideal conditions the bacterial colony counts on replicate plates will vary in the same manner as samples from a Poisson series. Accordingly, the index of dispersion, χ^2, was calculated for each set of parallel plates. The number of plates per set varied from twelve to three and, since the same value of χ^2 has different probabilities for different numbers of plates, the full presentation of the data here would require too much space. Instead, the values of χ^2 have been grouped so as to accord with Fisher's Table III (Fisher, 1938), and Table 1 shows the number of values of χ^2 which occur in each group. It is obvious that there is a large excess of high values of χ^2, where P is less than 0·01. But this same counting technique had previously been employed to count the bacteria in cultures grown in the same apparatus under similar conditions, except that no germicide was present, and it can be stated definitely that in a series of over 350 sets of replicate plates under the latter conditions there was no excess of very large values of χ^2. It is known that any significant departure from the theoretical distribution of χ^2 is an indication that the means calculated from the replicate colony counts may be wholly unreliable, and it is, therefore, important to know whether the values which are not excessive are distributed in accordance with expectation.

The details of the testing of the agreement of the 146 values remaining after excluding the ninety very high ones are shown in Table 1, and the agreement cannot be

Table 1. *Distribution of values of* χ^2 *for* 236 *sets of replicate plates in experiments with phenol as germicide*

Range of P	Numbers observed $(m+x)$	Numbers expected excluding the first group (m)	Difference (x)	x^2/m
< 0·01	90	—	—	—
0·01–0·02	2	1·475	+0·525	0·19
0·02–0·05	10	4·42	−5·58	7·04
0·05–0·10	11	7·37	+3·63	1·79
0·10–0·20	23	14·75	+8·25	4·61
0·20–0·30	5	14·75	−9·75	6·44
0·30–0·50	25	29·49	−4·49	0·68
0·50–0·70	25	29·49	−4·49	0·68
0·70–0·80	13	14·75	−1·75	0·21
0·80–0·90	15	14·75	+0·25	0·00
0·90–0·95	11	7·37	+3·63	1·79
0·95–0·98	4	4·42	−0·42	0·04
0·98–0·99	1	1·475	−0·475	0·15
> 0·99	1	1·475	−0·475	0·15
Totals	236			23·77

$n' = 13$; $P = 0{\cdot}022$ for $\chi^2 = 23{\cdot}77$.

considered satisfactory since $P = 0{\cdot}022$. The departure from expectation is largely due to an excess of values in the 0·1–0·2 group and a deficiency in the 0·2–0·3 group, since it happens that if these two groups are combined χ^2 is diminished by nearly 50 % and P rises to the satisfactory value of between 0·3 and 0·5. It is not possible to pursue this point further at the present time. Concerning the ninety excessively high values of χ^2, examination of their distribution amongst the experiments shows that 75 % of them occurred among the counts made when the mortality was greater than 95 %. In fact, 72 % of these counts were subject to excessive

variation, but only 16 % of counts made when the mortality was below 95 % were so affected. It may be concluded, therefore, that excessive variability appears when counts are made of bacteria which have been exposed for a considerable time to the action of phenol.

In Table 2 the results of typical experiments are given, all times being taken from the end of the 5 min. phenol addition period. The graphical presentation of these results is a difficult matter owing to the enormous range of population covered by each experiment, i.e. from over 300×10^6 organisms per ml. often to well below 50. Paper economy prevents their being presented in a variety of ways, as would have been desirable. In Figs. 2 and 3 the logarithms of the numbers of survivors are shown plotted against time, but it must be pointed out that in such a presentation the bulk of the mortality is compressed into a small space on each graph, although the behaviour of the relatively few highly resistant organisms is well emphasized. It is evident that the course of the disinfection changed markedly as the phenol concentration was altered.

The death-rate of a bacterial culture is here defined as $K = \frac{1}{t}(\log_{10} B - \log_{10} b)$, where B and b are the numbers of viable organisms at the beginning and the end of a period t, and K is obviously equal to the slope of the logarithm of survivors-time curve at any point. At low phenol concentrations there was a considerable period in which the death-rate was low but increasing, whereas at high concentrations this initial period was very short. Nevertheless, its existence, even at the highest concentrations used, is considered to be real. In taking all times of exposure from the end of the 5 min. phenol addition period it is tacitly assumed that no deaths occurred before all the phenol had been run in. But at 8 g. per l. this is unlikely to have been absolutely true, since in less than 4 min. the concentration must have reached 6 g. per l., a concentration which in itself

Table 2. *Numbers of survivors in* Bact. coli *cultures exposed to various concentrations of phenol*

Phenol conc. g./l.	Time min.	Survivors per ml.	Phenol conc. g./l.	Time min.	Survivors per ml.	Phenol conc. g./l.	Time min.	Survivors per ml.
8·00	0	354,200,000	4·62	10	323,900,000	3·96	0	326,500,000
	5	29,270,000		55	313,000,000		15	315,600,000
	17	1,007		100	273,000,000		70	295,700,000
	21	244		145	203,400,000		205	282,600,000
	25	20		200	37,660,000		325	300,100,000
	35	0		260	735,600		835	58,860
				315	6,866		900	5,038
6·98	0	331,970,000		375	1,095		1120	28
	30	235		435	34		1210	5
	35	21		525	0		1310	0
	40	13						
	45	0	4·25	10	346,300,000	3·76	10	342,000,000
				135	325,700,000		130	346,800,000
6·04	0	366,200,000		210	287,200,000		235	318,900,000
	8	267,100,000		270	228,100,000		325	302,100,000
	32	10,250,000		325	152,400,000		445	283,900,000
	58	6,306		385	60,870,000		580	185,400,000
	78	310		440	9,650,000		715	75,980,000
	98	16		505	1,160,000		1255	7,559
	118	0		565	67,785		1375	780
				620	2,733		1510	54
5·09	5	336,100,000		675	612		1630	5
	35	288,700,000		765	179		1735	0
	70	184,100,000		825	43			
	105	14,910,000				3·48	0	328,600,000
	140	106,900	4·00	5	330,900,000		1060	167,800,000
	180	2,847		45	325,500,000		1180	108,400,000
	225	356		115	309,700,000		1305	70,040,000
	270	67		170	301,800,000		1420	38,820,000
	315	10		230	304,000,000		1540	25,900,000
				290	269,500,000		1660	11,920,000
				350	242,000,000		1810	6,800,000
				405	203,700,000		2290	415,600
				465	134,100,000		2515	74,560
				515	74,280,000		2620	22,330
				600	12,900,000		2740	11,840
				675	2,490,000		2875	3,215
				1095	8			

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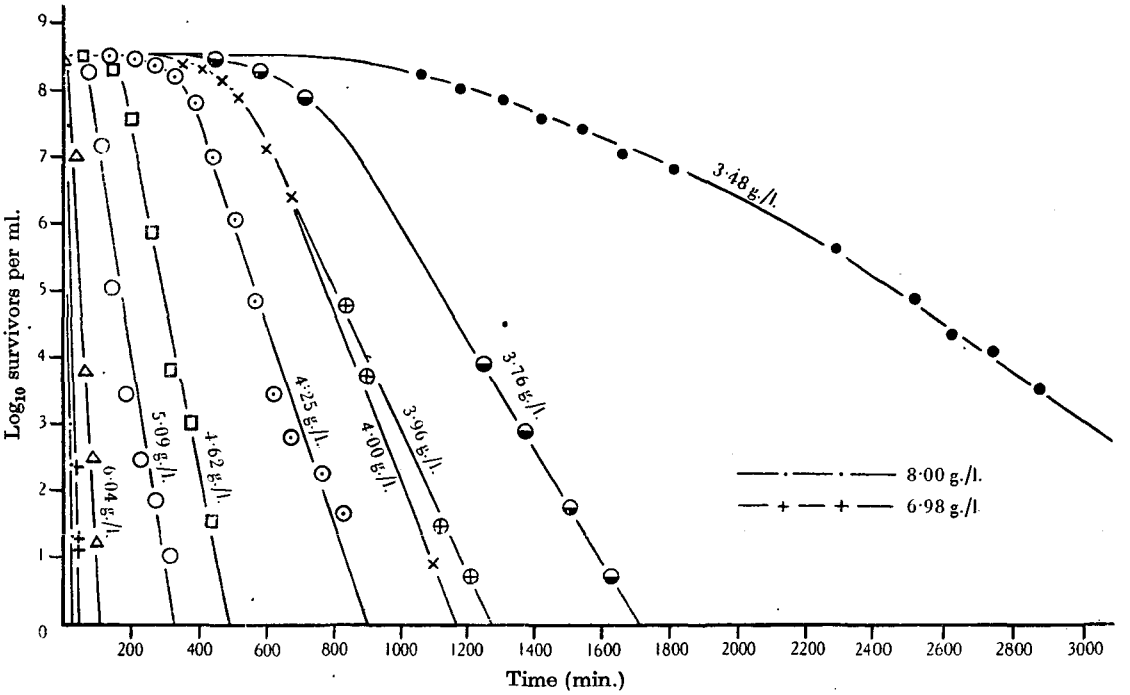

Fig. 2. Showing the relationship between logarithm of survivors and time for *Bact. coli* when exposed to various concentrations of phenol at 35° C.

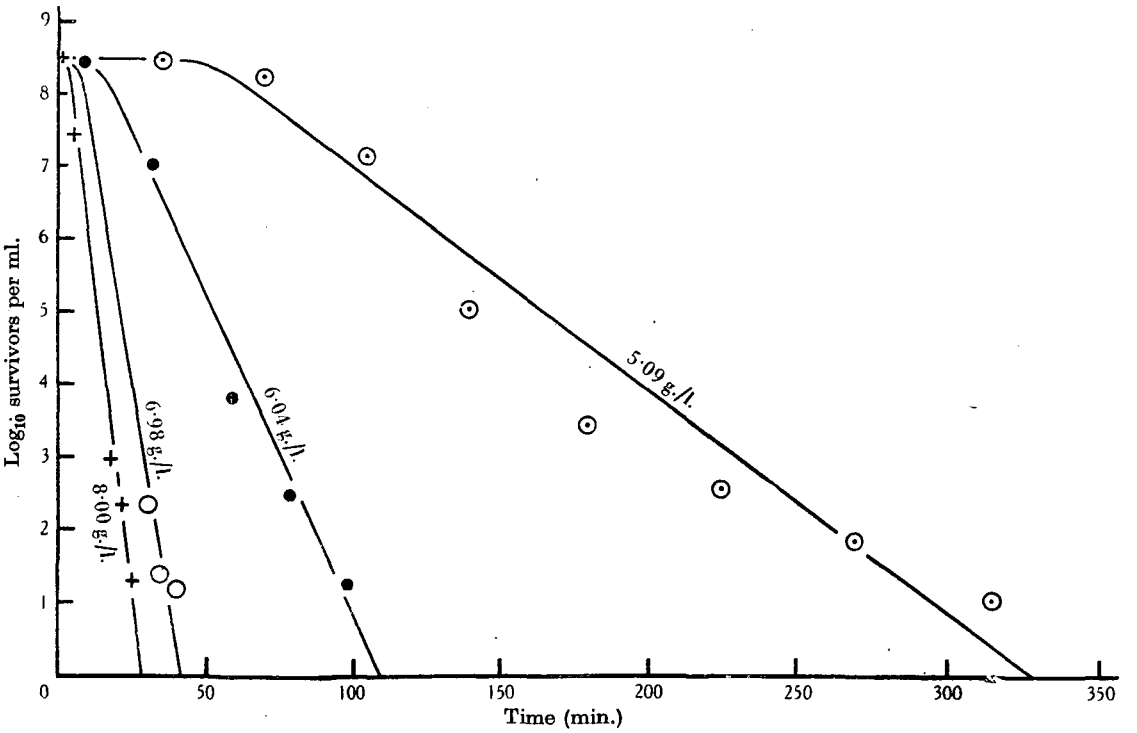

Fig. 3. Showing the relationship between logarithm of survivors and time for *Bact. coli* when exposed to various concentrations of phenol at 35° C.

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produced rapid death. The 5 min. allowance is, therefore, excessive at the highest concentration and the lag is artificially curtailed. Further argument, based on an analysis of the probit-logarithm of survival-time curves, in favour of the existence of a short lag phase even at 8 g. phenol per l. is set out below. The incidence of excessive variation in the plate counts does not affect this question of a lag phase, since this part of the disinfection process is wholly concerned with the period when the mortality was below 95 % (i.e. where the ordinates in Figs. 2 and 3 are greater than 7·3) except at the lowest phenol concentrations. In the graphs all the data have been plotted whether the values of χ^2 were excessive or not and, taking all the data, it would seem that in each experiment the death-rate rose to a maximum which was maintained until the disinfection process was virtually complete. At all events, straight lines can be fitted quite closely to the data in the later stages of each experiment, and the straight-line portions of the graphs in Figs. 2 and 3 are the calculated best straight lines whose formulae are given in Table 3. The 'virtual extinction time' and this is, obviously, the sum of Phases 1 and 2. The virtual extinction times, naturally, depend on the initial numbers of bacteria and for them to be strictly comparable with one another these initial numbers should have been constant. The mean initial bacterial population in millions per ml. was 338·67, with a standard error of ±4·27. This must be considered very satisfactory in view of the inevitable slight variations which must occur between different batches of nutrient medium. The virtual extinction times can, therefore, safely be used for the purpose of comparing the change in the relative durations of Phase 1 with rising phenol concentration (see Table 3). The variance of the virtual extinction time, $V(t)$, has been calculated from the formula $V(t)=\frac{V(\bar{y})}{b^2}+\frac{\bar{y}^2\,V(b)}{b^4}$, where $\bar{y}=\overline{\log_{10} S}$. The standard errors of the virtual extinction times varied between 1·5 % (at 3·48 g. phenol per l.) and 5·43 % (at 5·09 g. per l.) of the respective times with one exception, 0·15 % being obtained in the experiment at 3·76 g. per l.

Table 3. *Details of Phases* 1 *and* 2 *of the log survivors-time curves for* Bact. coli *exposed to various concentrations of phenol at* 35° C.

Phenol conc. g./l.	Regression formula of Phase 2 $\log_{10} S=\overline{\log_{10} S}+b\,(t-\bar{t})$, where S=no. of survivors per ml. and t=time in min.	Standard error of $\overline{\log_{10} S}$	Standard error of b	Virtual extinction time min.	Phase 1 (estimated) min.	Ratio of Phase 1 to virtual extinction time
8·00	$\log_{10} S=3·5381-0·3111\ (t-\ 17·00)$	±0·2220	±0·02967	28± 1·30	(1·5)	(0·054)
6·98	$3·3298-0·1941\ (t-\ 26·25)$	0·2083	0·01338	43 1·60	—	—
6·04	$4·5844-0·0847\ (t-\ 57·00)$	0·2162	0·006782	109 5·03	11	0·101
5·09	$4·1824-0·0301\ (t-\ 186·43)$	0·2656	0·003227	325 17·34	50	0·153
4·62	$4·3710-0·0254\ (t-\ 317·00)$	0·1723	0·002083	489 15·61	173	0·354
4·25	$4·4716-0·0146\ (t-\ 597·50)$	0·1615	0·001122	903 25·84	355	0·393
4·00	$5·5665-0·0123\ (t-\ 721·25)$	0·1281	0·0005742	1174 23·54	555	0·473
3·96	$2·6505-0·0107\ (t-1016·25)$	0·1075	0·0006992	1264 19·09	525	0·415
3·76	$2·2999-0·00850\ (t-1442·50)$	0·00979	0·0000694	1713 2·49	915	0·536
3·48	$4·4841-0·00359\ (t-2608·00)$	0·03251	0·0001631	3856 57·40	2245	0·582

ordinary method of least squares and the unweighted values of y (=$\log_{10}$ survivors) were used in the calculations. For convenience, the phase of increasing death-rate is hereafter referred to as Phase 1 and that of constant maximum death-rate as Phase 2. Usually, there was no difficulty in deciding, by inspection, which points belonged to the respective phases but when there was some doubt whether a point belonged to the end of Phase 1 or the beginning of Phase 2 it was found that its inclusion or exclusion from the latter made but little difference to the slope of the line or to its position. The ratios of the values of b to their standard errors varied between 9·5 and 22 to 1, with one exception where the ratio was 120 to 1.

The end of Phase 2 has been taken as the time at which there was one survivor per ml. as calculated from the (extrapolated) line of regression of log survivors on time by putting $y=0$ in the formulae of Table 3. The experiments were carried to such high mortalities that the extent of the extrapolation was slight. The whole duration of each experiment, i.e. the time taken to reach a survivor level of 1 per ml., has been called the

Clearly, the virtual extinction times decreased with increasing phenol concentration, as also did Phase 1, but the latter decreased more rapidly so that at about 7 g. per l. and over it was hardly detectable and the log survivors-time curves approximated to the straight-line form commonly regarded as characteristic of the decrease in numbers of viable bacteria in the presence of germicides. Also, the maximum value of the death-rate increased as the phenol concentration rose. The relations between phenol concentration, time for attainment of various degrees of mortality and maximum death-rate are fully discussed in the following paper of this series. Careful inspection of the curves in Figs. 2 and 3 and calculation of the death-rates between successive observations reveal that in the Phase 2 of the disinfection process which has been treated above as one of constant maximum death-rate there is, actually, a tendency for the rates to rise to a peak and then to decline. This 'peak effect' is shown particularly well in the experiments at 5·09 and 4·25 g. phenol per l. but it can be detected at all concentrations except the two lowest. This phenomenon is dealt with more fully below.

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Here it is necessary to answer an objection that may be raised on a point of technique. Broth, in small amounts, was added continuously throughout the experiments and it seemed possible that the death-rates observed were a reflexion of the changing medium or were affected by such changes. That this was not so was evident from two experiments conducted at exactly the same phenol concentration, but in one case the phenol was dissolved in excess Difco dehydrated broth solution. Neither the virtual extinction time, the duration of Phase 1, nor the maximum death-rate was affected by the additional organic matter.

DISCUSSION

There is no doubt that at low phenol concentrations, and when the bacteria are subjected to as little disturbance as possible apart from the introduction of the germicide, the death-rate of *Bact. coli* produced by phenol is far from being constant. To emphasize this fact Table 4 has been constructed which shows, for three representative concentrations, the rise in death-rate from the very low initial value until it reaches its maximum.

Table 4. *The variation of death-rate with time for* Bact. coli *exposed to three representative phenol concentrations at* 35° C.

Time interval min.	Death-rate per min.
3·76 g. per l.	
10– 130	0·0
130– 235	0·00028
235– 325	0·00026
325– 445	0·00022
445– 580	0·00137
580– 715	0·00287
715– 915	0·00509
915–1713	0·00850
4·62 g. per l.	
10– 55	0·0003
55–100	0·0013
100–145	0·0028
145–200	0·0133
200–489	0·0254
5·09 g. per l.	
5– 35	0·0022
35– 70	0·0056
70–326	0·0301

The regular and clear-cut nature of the results here reported has afforded ample reward for the somewhat elaborate technique employed. Previously, there have been indications from the work of others, e.g. Chick (1910), Cohen (1922), Levine, Peterson & Buchanan (1928), Myers (1928, 1929), Hobbs & Wilson (1942), that bacteria do not always follow the so-called logarithmic order of death, but it has usually been the case that, in addition to being subjected to the action of the germicide, the test organisms have been otherwise ill-treated, for example, by suddenly being transferred from a warm nutrient solution into cool non-nutritive fluid. The observed death-rates, therefore, might well have been those of weakened organisms and not those of healthy bacteria. Recently Withell (1938), also working with *Bact. coli* and phenol, obtained results somewhat similar to those reported here, but he considered that his results showed that at low phenol concentrations an initial low constant death-rate was fairly suddenly replaced by a higher, but also constant, death-rate. In the present work, Phase 1, whenever its duration was long enough to allow observations to be made, showed not a constant but an increasing death-rate which gradually merged into the constant death-rate of Phase 2.

It is necessary to consider whether the death of the organisms began immediately after the addition of the phenol or whether there was an initial period of 'no death'. It is a matter of some importance in the study of the dynamics of disinfection whether or not there is any such period. From Table 4 it appears that at low phenol concentrations the death-rate was exceedingly small for a considerable time and it is possible that at very low concentrations a period of 'no death' does exist. The exact shape of the survivors-time curves during the early part of Phase 1 is, however, uncertain for the reason that each count was subject to an error of random sampling from the final dilution as well as to a volume error introduced in making the dilutions. Even if the latter error had been negligible, the former, with eight replicate plates and a mean number of colonies of 275 (the most frequent values for the opening stages of an experiment), would have amounted to 2·2 %. Still neglecting the volume error, the smallest percentage kill that could be regarded as significant ($P = 0{\cdot}05$) would be 6·5 % or a mortality of about 22×10^6 bacteria per ml. The possibility of the existence of a period of 'no death' cannot, therefore, be denied at present. It is hoped later to conduct further experiments and to secure greater accuracy in order to define more closely the possible extent of this period. It is anticipated, in view of the fact that the subsequent counts have almost always been less than the initial count, even after comparatively short exposures to phenol, that the more accurate data will show that the possible extent of the 'no death' period is much shorter than the present data entitle one to believe.

Further work is also needed before the shape of the latter parts of the disinfection curves can be regarded as firmly established. This will have to take the form of an endeavour to improve the counting technique, so that the excessive variation among replicate plates, characteristic of the later stages of the experiments, is eliminated. It may not be possible to do this, since the evidence suggests that it is an inherent property of bacteria which have been exposed to an injurious concentration of phenol for so long that 95 % of them have succumbed. Withell (1938) also noted this excessive variation in replicate plate counts, but in his case the technique was such that it also appeared among counts of bacteria which had not been exposed to a germicide. This author did not carry his experiments to such high mortalities as those reported here and his published data (1938) contain few values above 95 % mortality and none above 99·1 %. In our experience the bulk (75 %) of the excessive variation occurred when the mortality

had exceeded 95 %, and Withell's data exhibited a similar trend, for examination of the 40 counts made on *Bact. coli* exposed to phenol and set out in detail by him, shows that 18 were excessively variable, and of these 16 occurred after the mortality had reached 50 %, out of a total of 21 counts made in that period.

Another body of detailed data is provided by Withell (1942). When this is subjected to statistical analysis it appears that out of a total of 138 five-plate counts of a species of *Micrococcus* exposed to either phenol or *para*chlor*meta*cresol, no less than 58 or 42 % were excessively variable ($\chi^2 > 9{\cdot}488$, $P < 0{\cdot}05$), although the mortality rarely exceeded 95 %. The excessive variation was not confined to mortalities exceeding 50 % and in the experiments with phenol the proportion of excessively variable counts was about the same (37 %) both above and below that level of mortality. With *para*chlor*meta*cresol, however, the incidence of excessive variation was 27 % for mortalities below 50 % but 55 % amongst counts made at higher mortalities. The attainment of a statistically perfect counting technique is evidently no simple matter, even when the cells are those of a single species of bacteria. Organisms exposed to a germicide for a considerable time appear to need far more careful treatment than those not so exposed or exposed for short periods only. It is satisfactory to find that the technique employed in the present research has prevented excessive variation from appearing before the late stages of the experiments, yet its existence, even though largely confined to a particular stage of the experiments, must cause any calculations or deductions involving that phase to be treated with reserve. In particular, reference has been made above to a tendency, indicated at all concentrations except the two lowest, but especially noticeable at 5·09 and 4·25 g. phenol per l., for the death-rate not to remain constant at its maximum value but to rise to a peak and then to decline. Now, the part of the disinfection process which is open to this interpretation lies wholly in the region where the mortality was greater than 95 %, i.e. where excessive variation was prominent. It so happens that such excessive variation was very pronounced at the two concentrations where the peak effect was most marked. If these two cases are omitted from consideration, the remainder contain less convincing evidence of peaks and the death-rates in their later stages might well be scattered around means. Further, it is true that if all the counts with excessive values of χ^2 are eliminated, the peak effect is no longer detectable in any experiment where sufficient points remain for a series of death-rates to be calculated. It is of interest to note here that Hobbs & Wilson (1942) observed a peak in the death-rate in their experiments with *Bact. coli* exposed to caustic soda. Unfortunately, technical difficulties prevented them from demonstrating the final decline in death-rate when the general death-rate was rapid. In the following treatment of the present data the excessive variation among plate counts has been disregarded because of the interesting nature of the deductions which can be made from the observations as a whole, but certainly many of the points which fit least well into the general scheme outlined could well have been rejected on the score of excessive variation.

In the past many attempts have been made to account for the peculiarly skew distribution of the survival times of bacteria exposed to lethal agencies, required by the so-called constant logarithmic death-rate of the organisms. In spite of the difficulties of its demonstration, it is hard to dismiss the idea that the survival times of bacteria, as of other organisms, are in reality distributed normally. Possibly, the apparently very skew distribution of the survival times results from experimental methods involving the use of too powerful germicides, or rough handling of the cells, whereby the less resistant organisms are swamped and the survivors-time curves become exponential. The normal distribution of survival times might then be obtained using low phenol concentrations and with careful handling of the cells. The series of experiments reported above was designed, in part, to test this idea, and it is important to remember that special care was taken to ensure that as far as possible the bacterial populations were completely reproduced in each experiment. For a hypothetical population of cells whose survival times are distributed normally, the symmetrical sigmoid survivors-time curve becomes transformed to one of continually increasing slope when logarithms of survivors are plotted against time. The rate of increase of slope at first, and towards the end, is slow and in effect the beginning and end portions of the curve approximate to straight lines. In Fig. 4 the theoretical curves for two such populations are shown. The times of 50 % mortality and virtual extinction are those found empirically for the lowest concentrations of phenol used, viz. 3·48 and 3·76 g. per l. The circles in the figure indicate the actual experimental points, and it is obvious that the experimental log survivors curves approximate to the ideal curves calculated for the hypothetical populations of cells whose survival times are distributed normally. In Fig. 5 the distributions of survival times for three phenol concentrations are shown in the form of histograms and it is evident that, even under the special experimental conditions employed, identical populations still show very skew distributions of survival times when exposed to high phenol concentrations, even though the distributions become more nearly normal at low concentrations. The fact that these skew distributions do become more normal when slow death-rates permitting observations of low mortalities are in operation, is some justification for assuming that skew distributions may be artifacts, due partly to technical difficulties in observing small mortalities when death-rates are high (at 8 g. phenol per l. 90 % of the organisms died in less than 3 min.). Further, in this investigation, changes in the slope of the logarithms of survivors-time curves are far more marked than any previously obtained by other workers, a result which is attributed to the avoidance of any alteration in the environment of the cells other than by the inclusion of the germicide. This is in accordance with the view that procedures which involve radical changes in the cells' environment, such as alteration in temperature and changes in the concentration and composition of the surrounding fluid, may so affect the cells than an inherently normal, or nearly normal, distribution of survival times becomes highly skewed.

The data are not in a form suitable for making the direct comparison, by the usual methods, with the results to be expected on the assumption that the

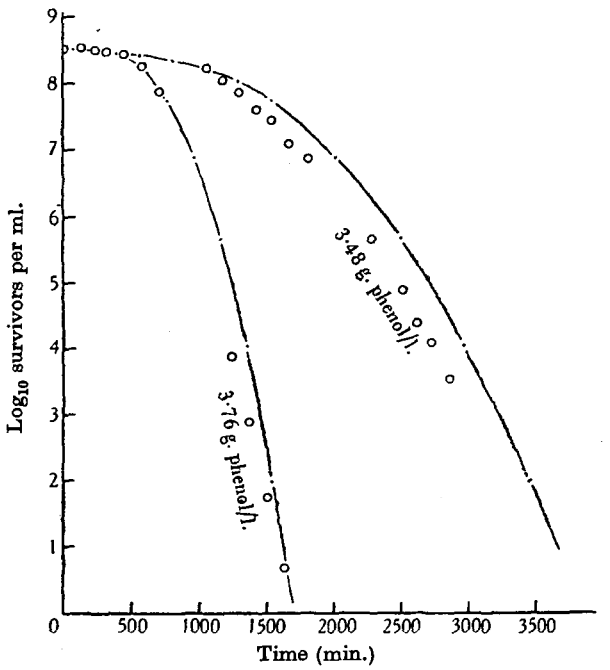

Fig. 4. Theoretical curves showing relationship between the logarithm of survivors and time for two hypothetical populations whose survival times are distributed normally (see text). Circles indicate experimental values for *Bact. coli*.

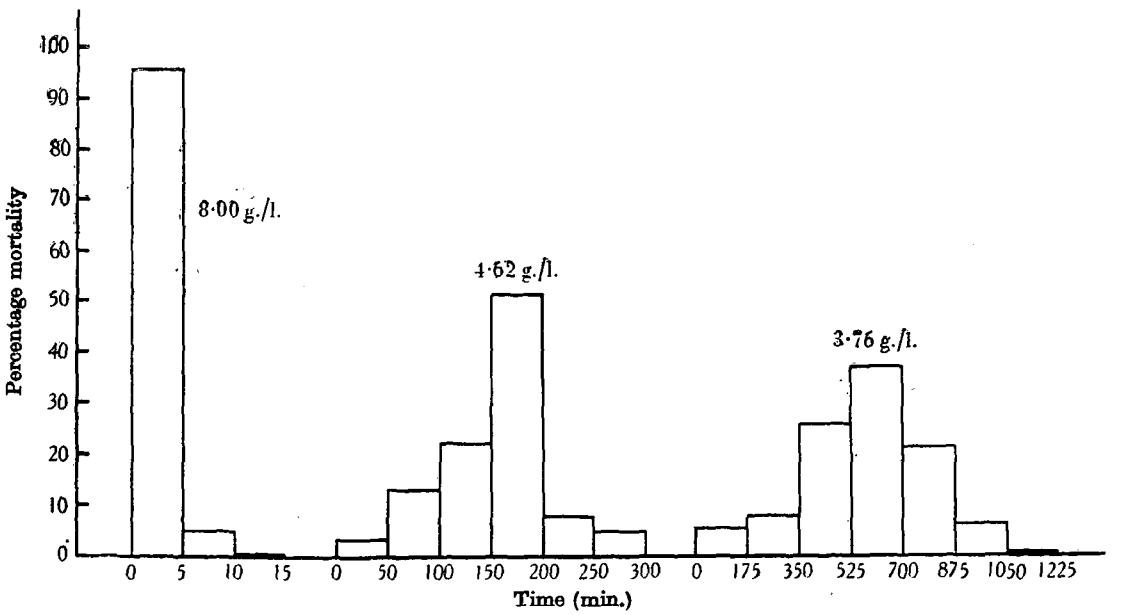

Fig. 5. Showing distribution of mortality of *Bact. coli* at three phenol concentrations, with respect to time.

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survival times are distributed normally, but use may be made of the 'normal equivalent deviations' of Gaddum (1933) or the probits of Bliss (1935). Tables for the conversion of percentage mortalities into probits are given by Bliss (1935, 1938), but these do not cover mortalities greater than 99·99 %, and for the higher mortalities encountered in the present work use has been made of Pearson's Table II (Pearson, 1930). In Table 5 are set out the percentage mortalities for the present experiments, and their probit values, in relation to times of survival and phenol concentration. When probits are plotted against times of survival (Fig. 6), a set of curves which approach more nearly to straight lines as the phenol concentration is reduced is obtained. The general shape of each curve is that of two arms of small slope joined by a central steeper portion, and the approximate linearity at low concentrations arises through a lessening of the slope of the latter.

Bliss (1937) examined cases of higher organisms whose resistances to lethal agents were distributed normally when measured in terms of the log survival times, and it appeared desirable to examine the present data from this point of view. Fig. 7 shows the result of plotting probits against logarithms of survival times (data in Table 5). There are here strong indications that at low concentrations of phenol two intersecting straight lines fit the points best, the change of slope being well marked and abrupt. As the phenol concentration rises the slope of the lower line increases while that of the upper tends to diminish, so that the point of intersection rises on the ordinate and the whole graph tends to approximate to a single straight line. To establish this point firmly more information is needed in the region of probit 4–6 (16–84 % mortalities) at the higher concentrations. Generally, the graphs in Fig. 7 are more nearly linear (even though bi-linear) than those

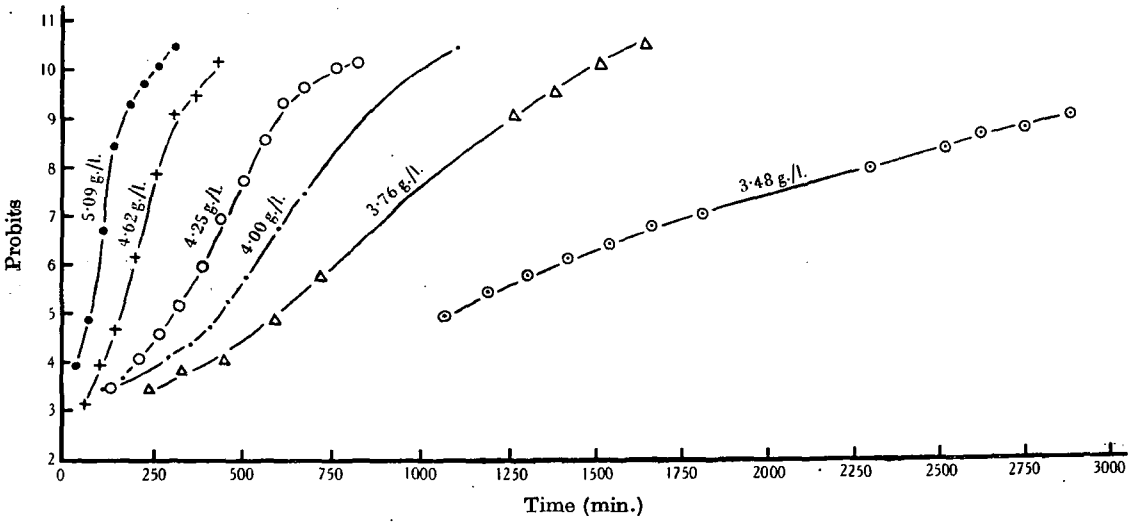

Fig. 6. Showing relationship between probits and time of survival of *Bact. coli* at various phenol concentrations.

Even in the more curved lines there is a portion, extending from probit 5 (or slightly below) to 8, which approximates to linearity. This corresponds to mortalities of from 50 to 99·87 %. However, it is not until the concentration is reduced to 3·76 g. per l. that the approximate linearity extends down to 4 probits. This figure corresponds to about 16 % mortality and the range 4–8 probits covers about 84 % of the organisms. It may be said, therefore, that under the special experimental conditions employed between 50 and 84 % approximately (according to phenol concentration) of the bacterial cells showed a nearly normal distribution of survival times. The hypothesis of a fundamentally normal distribution of survival times, which is usually skewed artificially, is thus supported.

It is possible that on some basis other than that of the survival time the resistances of the cells may show a closer approach to a normal distribution. For determining this the probit method is especially useful. in Fig. 6. In particular, on the logarithmic time basis the relatively few highly resistant organisms fall into line with the remainder in a very satisfactory manner especially at low phenol concentrations. The feature of two intersecting straight lines is puzzling. However, in dosage-mortality data it seems to be well known. Bliss (1935) states that for multicellular organisms '. . . this change in slope at a kill of about 33 % is a frequent phenomenon. . . ' and he discusses it in relation to the percentage absorption of the drug. The present observations give, to the best of our information, the first clear evidence of its existence in bacteriological time-mortality data although Withell (1942) makes a brief reference to its appearance in his results, where it was, however, not strongly marked. The same author also converted the time-mortality data of Hewlett (1909) on the death of mustard seeds in 0·2 % mercuric chloride and those of Oothuisen (1935) on the death of the confused flour beetle at 44° C. into probit-logarithm

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Table 5. *Percentage mortalities of* Bact. coli *and their probit values in relation to times of survival at different phenol concentrations at* 35° C.

Phenol conc. g./l.	Time min.	Log_{10} time	Percentage mortality	Probit
3·48	0	—	0	—
	1060	3·0253	48·94	4·9733
	1180	3·0719	67·01	5·4402
	1300	3·1139	78·69	5·7956
	1420	3·1523	88·19	6·1843
	1540	3·1875	92·19	6·4179
	1660	3·2201	96·37	6·7956
	1810	3·2577	97·93	7·0398
	2290	3·3598	99·87	8·0549
	2515	3·4006	99·977	8·5109
	2620	3·4183	99·9932	8·8154
	2740	3·4378	99·9964	8·9694
	2875	3·4586	99·999022	9·2704
3·76	10	1·0000	0	—
	235	2·3711	6·73	3·5041
	325	2·5119	11·67	3·8085
	445	2·6484	16·99	4·0452
	580	2·7634	45·79	4·8943
	715	2·8543	77·78	5·7649
	1255	3·0986	99·99779	9·0844
	1375	3·1383	99·99977188	9·5840
	1510	3·1790	99·99998415	10·1131
	1630	3·2122	99·99999855	10·5475
4·00	5	0·6990	0	—
	45	1·6532	1·63	2·8634
	115	2·0607	6·41	3·4785
	170	2·2304	8·81	3·6474
	290	2·4624	18·56	4·1056
	350	2·5441	26·87	4·3831
	405	2·6075	38·43	4·7057
	465	2·6675	59·47	5·2395
	515	2·7118	77·55	5·7571
	600	2·7782	96·10	6·7625
	675	2·8293	99·25	7·4314
	1095	3·0395	99·99999766	10·4633
4·25	10	1·0000	0	—
	135	2·1303	5·95	3·4408
	210	2·3222	17·07	4·0485
	270	2·4314	34·13	4·5912
	325	2·5119	56·01	5·1511
	385	2·5855	82·43	5·9317
	440	2·6435	97·22	6·9134
	505	2·7033	99·66634	7·7128
	565	2·7520	99·98043	8·5459
	620	2·7924	99·999211	9·3175
	675	2·8293	99·99982333	9·6371
	765	2·8837	99·99997734	10·0452
	825	2·9165	99·99998753	10·1582
4·62	10	1·0000	0	—
	55	1·7404	3·37	3·1716
	100	2·0000	15·73	3·9945
	145	2·1614	37·21	4·6737
	200	2·3010	88·37	6·1939
	260	2·4150	99·773	7·8379
	315	2·4983	99·9979	9·0944
	375	2·5740	99·99966187	9·5010
	435	2·6385	99·99998942	10·1888
5·09	5	0·6990	0	—
	35	1·5441	14·12	3·9251
	70	1·8451	45·22	4·8800
	105	2·0212	95·57	6·7024
	140	2·1461	99·9682	8·4173
	180	2·2553	99·99915307	9·3008
	225	2·3522	99·99989424	9·7421
	270	2·4314	99·99998005	10·0694
	315	2·4983	99·99999717	10·4294
6·04	0	—	0	—
	8	0·9031	27·07	4·3893
	33	1·5185	97·20	6·9113
	58	1·7634	99·9983	9·1426
	78	1·8921	99·99991538	9·7871
	98	1·9912	99·99999573	10·3554
8·00	0	—	0	—
	5	0·6990	91·74	6·3876
	17	1·2304	99·99971578	9·5378
	21	1·3222	99·99993124	9·8286
	25	1·3979	99·99999441	10·3065

of time curves. In the former case it is obvious that two intersecting straight lines fit the data very well and in the latter there is an indication of the same condition. Withell regards the line of lesser slope as unimportant, on the ground that it related only to a small proportion of the organisms. But, however small that proportion may have been in Withell's own experiments, in those of Oothuisen it may have amounted to nearly 50 %, while in those reported here it was never less than 21 % (probit 4·2) and tended to increase with rising phenol concentration.

When the probit-log time graph is a straight line the logarithms of survival times are distributed normally. The slope of the line is the reciprocal of the standard deviation of the log survival times. It can, therefore, be said that at low concentrations of phenol the distribution of log survival times is such that a portion of a normal curve with a larger standard deviation would fit those of the less resistant organisms, while a portion of another normal curve with a smaller standard deviation would fit the remainder. At low concentrations the less resistant organisms comprised about 20 % of the

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total. This proportion appeared to grow larger as the phenol concentration rose, while the standard deviations respectively decreased and increased so that the portions of normal curves of different standard deviations seemed ultimately to become parts of a single curve. Alternatively, the change in slope of the probit-log time graphs could have been due to the combination of two normal populations in unequal numbers, the smaller having a lower mean survival time (but possibly the same standard deviation) as the larger. Assuming the bacterial populations to have been homogeneous, it seems that at low phenol concentrations the survival times show a nearer approach to a normal distribution than the log survival times, but it is possible that at the higher concentrations the reverse is true. But if it be admitted that the bacterial populations may have been mixtures in unequal proportions of two populations of differing mean survival times then, in view of the striking linearity of the graphs in Fig. 7, it would seem right to conclude that the logarithms of survival times show the closer approach to a normal distribution at all phenol concentrations. No attempt has yet been made to employ statistical methods for testing how nearly the points in Figs. 6 and 7 fit straight-line graphs and space will not permit a graphical comparison of the data with hypothetical curves. However, probit values very much higher than those obtained by Withell (1942) have been recorded and the linearity in these upper regions on the probit-log time graphs has been well maintained.

Withell (1938) and others previously have found that a variation in type of response occurs when the strength of the bactericide is altered, log survivors-time curves showing lag phases at low concentrations but becoming straight lines at high concentrations. The present work indicates that this variation is more apparent than real since, under the special experimental conditions employed, there was evidence of the existence of a short lag period even at a phenol concentration of 8 g. per l. Irwin (1942) has stressed the point that when the true law is exponential it would require very good data to distinguish between that law and the hypothesis of a normal distribution of the logarithms of survival times. But it is true that if the logarithms of the survival times of a homogeneous population are distributed normally then the corresponding curve of log survivors plotted against time must show a lag. It may be small, since it depends on the standard deviation, and be difficult to detect experimentally. This is illustrated in Fig. 8 where the theoretical straight probit-log survival-time line *A* (inset) has as its positions of 50 % mortality and virtual extinction time (1·9 and 28·4 min., respectively) those indicated by the experimental data at 8·00 g. phenol per l. The corresponding theoretical log survivors-time curve *A* shows a short lag. However, the curve runs nearer the ordinate at zero time than the experimental points which are shown in the diagram by circles. This is understandable because, as mentioned earlier, all times are taken from the end of the 5 min. occupied by the addition of the phenol solution and,

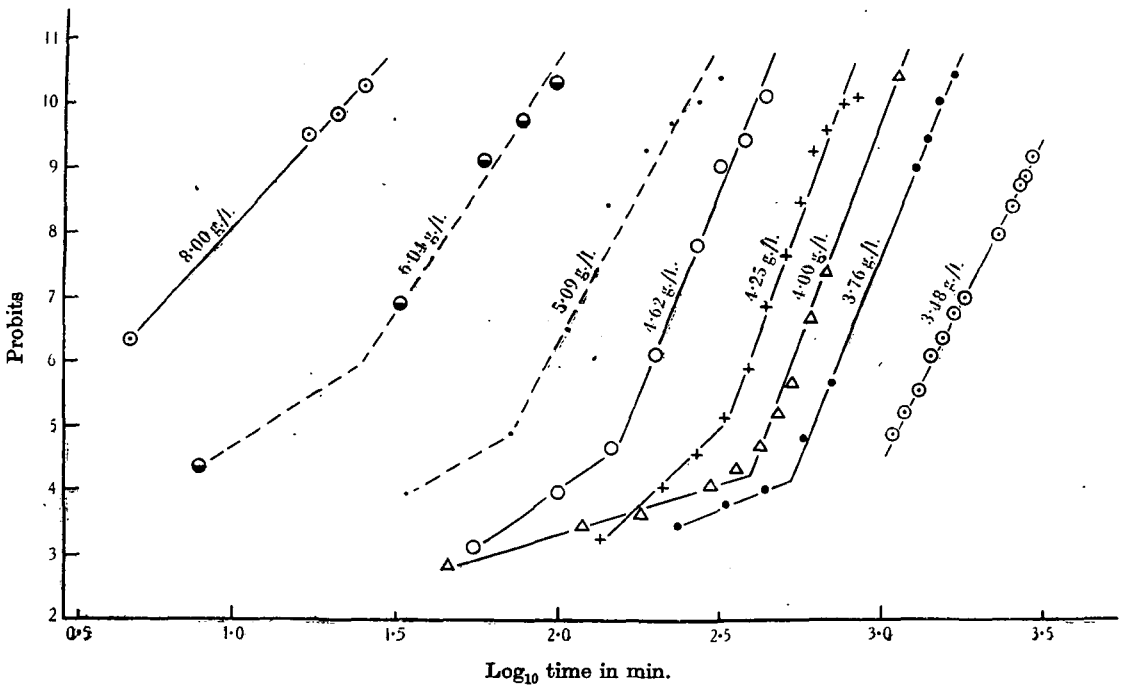

Fig. 7. Showing relationship between probits and logarithm of survival times for *Bact. coli* at various phenol concentrations. The two broken-line graphs are drawn so as to form a link between the graphs for the lower and higher phenol concentrations.

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at this concentration, death probably began before all the phenol had been added. In less than 3 min. the phenol concentration would already have risen to 6 g. per l. and this concentration produced a 27 % mortality in 8 min. At 8 g. per l., therefore, the times to reach 50 % kill and 1 survivor per ml. are very probably underestimated. Curve *B* in Fig. 8 is a hypothetical curve based on a 50 % kill at 3 min. and a virtual extinction time of 30 min. and the experimental data agree very well with this. Yet the lag period indicated by curve *B* is still only in the region of 2 min. and would be difficult to detect experimentally.

The above results can be changed into the dosage-mortality form by obtaining from the smoothed survivor-time curves the mortalities after fixed times of exposure to various phenol concentrations. When these mortalities are converted to probits and plotted against the dosages, curves are obtained (Fig. 9, data in Table 6) all of which are, probably, parts of sigmoid curves. These data are not very satisfactory, not only because the material has been obtained from smoothed curves instead of being actual observations, but also because from the nature of the results and the range of concentrations, five points at most can be secured for any one dosage-probit curve. The data are scanty in the important 4–6 probit range. However, there are indications that for short exposure times, i.e. up to 50 min., more than half the organisms (the 68 % covered by the 4–6 probit range) show a normal distribution of resistance when that is assumed to be proportional to the dosage, though more data are plainly desirable. Also, the more resistant half of the population (represented by the probits above 5) appears to have the same property for longer

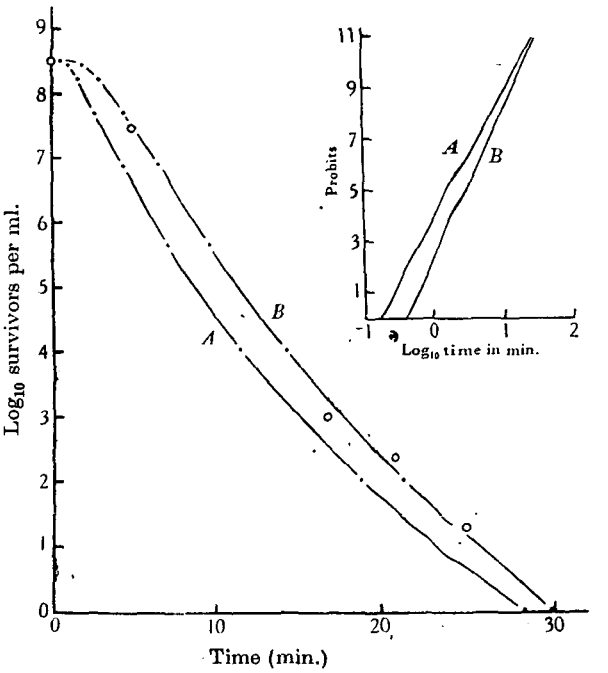

Fig. 8. Showing the effect of transforming straight probit-log survival time graphs (inset) to log survivors-time curves (see text). Circles indicate experimental values for *Bact. coli* at 8·00 g. phenol per l.

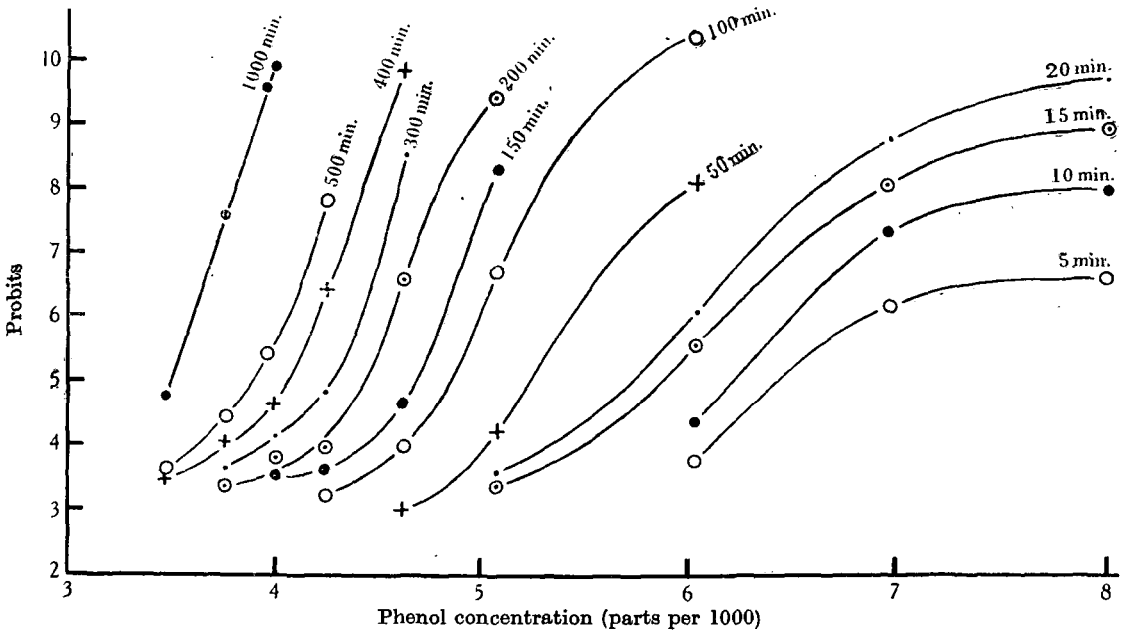

Fig. 9. Showing relationship between probits and phenol concentration for various exposure times of *Bact. coli*.

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Table 6. *Relation between percentage mortality of* Bact. coli *and phenol dosage with corresponding probit values, for various exposure times, at* 35° C.

Exposure time min.	Dosage g./l.	Log_{10} dosage	Percentage mortality	Probit
5	6·04	0·7810	10·5	3·7464
	6·98	0·8439	88·1	6·1800
	8·00	0·9031	94·73	6·6192
10	6·04	0·7810	27·1	4·3900
	6·98	0·8439	98·92	7·2973
	8·00	0·9031	99·8646	7·9993
15	5·09	0·7067	4·6	3·3151
	6·04	0·7810	69·94	5·5225
	6·98	0·8439	99·90	8·1007
	8·00	0·9031	99·9959	8·9400
20	5·09	0·7067	6·4	3·4780
	6·04	0·7810	85·73	6·0683
	6·98	0·8439	99·9913	8·7534
	8·00	0·9031	99·99988634	9·7275
50	4·62	0·6646	2·2	2·9859
	5·09	0·7067	21·8	4·2210
	6·04	0·7810	99·89	8·0649
100	4·25	0·6284	3·7	3·2134
	4·62	0·6646	15·5	3·9848
	5·09	0·7067	95·44	6·6891
	6·04	0·7810	99·99999366	10·2835
150	4·00	0·6021	7·7	3·5745
	4·25	0·6284	8·7	3·6406
	4·62	0·6646	37·2	4·6734
	5·09	0·7067	99·95	8·2711
200	3·76	0·5752	5·1	3·3648
	4·00	0·6021	11·3	3·7893
	4·25	0·6284	15·0	3·9636
	4·62	0·6646	93·8	6·5390
	5·09	0·7067	99·99936926	9·3666
300	3·76	0·5752	9·0	3·6592
	4·00	0·6021	19·4	4·1367
	4·25	0·6284	42·0	4·7981
	4·62	0·6646	99·98	8·5118
400	3·48	0·5416	6·0	3·4452
	3·76	0·5752	16·0	4·0055
	4·00	0·6021	35·5	4·6281
	4·25	0·6284	92·1	6·4118
	4·62	0·6646	99·99991742	9·7920
500	3·48	0·5416	8·8	3·6468
	3·76	0·5752	30·0	4·4756
	3·96	0·5977	65·0	5·3853
	4·00	0·6021	70·5	5·5388
	4·25	0·6284	99·73	7·7809
1000	3·48	0·5416	40·0	4·7467
	3·76	0·5752	99·56	7·6229
	3·96	0·5977	99·99976894	9·5813
	4·00	0·6021	99·99995288	9·9008

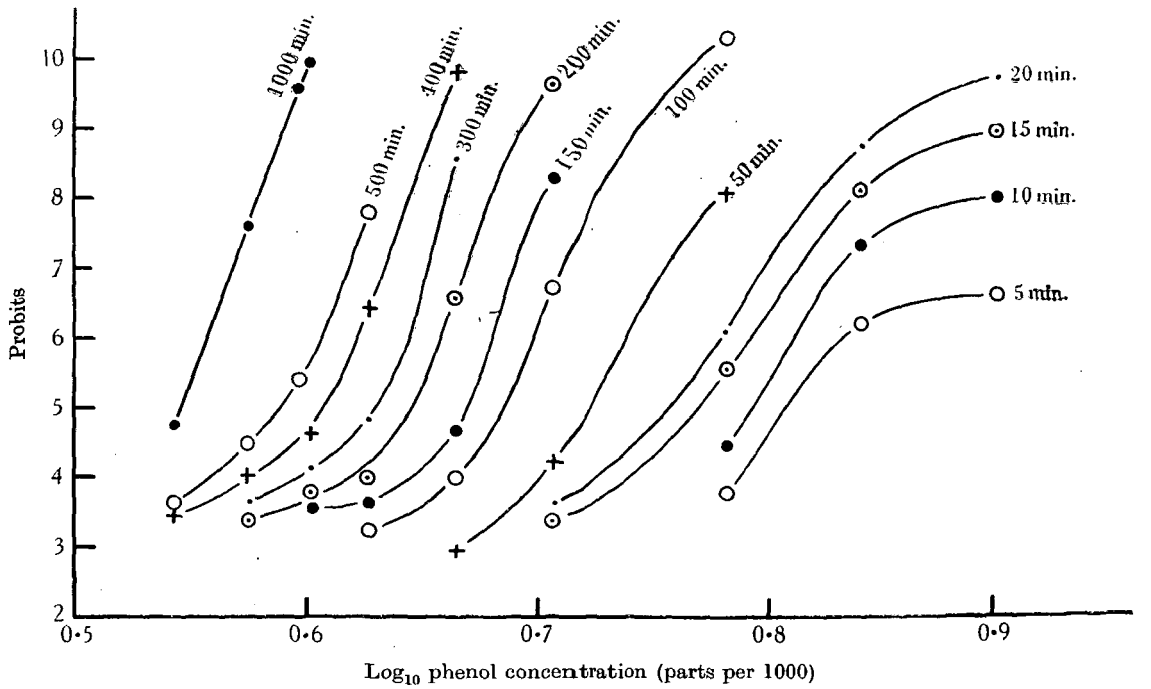

Fig. 10. Showing relationship between probits and the logarithm of phenol concentration for various exposure times of *Bact. coli*.

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exposure times. Fig. 10 shows the result of plotting probits against the logarithms of the dosages. Generally speaking, the curves are similar to those of Fig. 9. Data are again scanty in the 4–6 probit range where for short exposure times the probit-log dosage curves appear to be approximately linear, though they are not as convincingly so as are the comparable probit-dosage graphs. For longer exposure times the probit-log dosage curves resemble the bi-linear form of the probit-log survival-time curves and, moreover, they show a gradual straightening with increasing time analogous to that shown by the probit-log time curves with increasing concentration.

SUMMARY

1. The action of phenol on *Bact. coli* has been studied quantitatively in detail under such conditions that unfavourable circumstances other than the presence of the germicide were as far as possible eliminated. Briefly, this involved the addition of phenol solution directly to a large volume of culture developed under rigidly standardized conditions.

2. When the mortality exceeded 95 % the variation between the numbers of colonies on replicate plates, when determining the numbers of survivors, was often excessive.

3. Under the conditions used the death-rate was not constant. First, there was a phase (1) of slow but increasing death-rate which merged gradually into a phase (2) of approximately constant rate which was also the maximum for a given phenol concentration. There are indications that the death-rate may decline towards the extreme end of the disinfection process.

4. The virtual extinction time (the time taken for the survivors to fall to 1 per ml. as determined by extrapolation of the log survivors-time curve) increased as the phenol concentration decreased, as also did the duration of phase 1. The latter occupied about 60 % of the virtual extinction time when the concentration was 3·48 g. phenol per l., but with rising concentration its duration decreased at a greater rate than the virtual extinction time so that at about 7 g. per l. phase 1 was very short though still detectable. The standard errors of the virtual extinction times varied between ± 1·5 % and ± 5·43 %.

5. Using the probit method it has been shown that at low concentrations of phenol the distribution of resistance of *Bact. coli* is approximately normal when the resistance is measured in terms of the survival times.

6. The distribution of resistance was found to be normal at all concentrations of phenol used, when that resistance was measured in units of the logarithms of the survival times, with the exception that at the lower concentrations there was a sudden change in the slope of the probit-log survival-time line at about 20 % mortality. This change became less marked with rising concentration and was probably absent at 8·00 g. phenol per l.

7. After conversion into the dosage-mortality form, the data reveal that about 70 % of the cells show an approximately normal distribution of resistance when the latter is expressed in terms of the dosage survived for a fixed time, provided that the exposure time is less than 50 min.

8. The distribution tends to be normal for longer exposure times when the resistance is measured in terms of the logarithms of the dosages survived. On this basis a change of slope of the probit-log dosage line is observed as in the case when resistance is measured in terms of the logarithms of survival times.

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