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SYMPOSIUM

CHEMICAL DISINFECTION

SOME ASPECTS OF THE DYNAMICS OF DISINFECTION

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THE course of the destruction of populations of bacteria by chemicals, and other lethal agents, can be followed by taking samples at intervals and determining the numbers of survivors by making colony counts on a suitable nutrient medium. With chemical disinfection it is necessary to ensure that the action of the chemical should be completely stopped when the sample is taken, either by the use of a neutralising agent or by dilution, for if this is not done there will be continuing action and the mortality obtained will be greater than that which existed at the moment of sampling. Here it is relevant to mention that nutrient media used for the cultivation of bacteria may contain substances which are lethal to cells already weakened, though not irreversibly damaged, by exposure to phenolic and other antiseptics, as shown by Jacobs and Harris[1] and Richards[2]. However, any errors which are thereby introduced are normally small and may be disregarded.

The idea that the survival curves of bacterial populations exposed to chemicals and other lethal agents could be expressed adequately in the form of straight lines by plotting the logarithm of the number, or the percentage, of survivors against time is of respectable antiquity, as it was first put forward in 1907 by Madsen and Nyman[3]. Instances where the experimental data deviated from the straight line were soon discovered; but these were usually explained away in terms of the presence of clumps of cells, or of populations of differing sensitivity in the supposedly homogeneous initial populations, and the ideal or true expression of disinfection data was held to be the straight line on this semilogarithmic graph.

In these circumstances it was not surprising that attention should be drawn to the similarity between the rate of a disinfection and that of a so-called unimolecular chemical reaction, where the "logarithmic law" also applies, and inevitably the analogy was pushed to its logical conclusion, which was that viable bacterial cells each contain a single molecule whose destruction or alteration results in the death of the cell. This idea was very strongly upheld by Rahn[4] as recently as 1943, but it was quite unsupported by direct evidence and it always appeared unlikely that so

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great a diversity of chemical agents could all act on the one molecule. Indeed, modern knowledge of the mode of action of disinfectants has made the theory wholly untenable and there is now no compulsion to believe that the "logarithmic law" must apply rigidly and in all circumstances. In fact, careful studies by various workers, including Withell[5] and Jordan and Jacobs[6,7], have revealed that a variation in type of reponse apparently occurs when the overall rate of the reaction is altered by changing the concentration of disinfectant or the temperature at which it acts, so that whereas in fast reactions the graph of log survivors against time is approximately a straight line, with slower and slower reactions there is an increasingly prominent initial phase of relatively low death rate. But Jordan and Jacobs[7] concluded that the variation was not a real one. Their analysis of a considerable body of data derived from an extensive study of the action of phenol on standard cultures of *Escherichia coli* under carefully controlled conditions suggested that the shape of the semilogarithmic disinfection curve was fundamentally the same in all circumstances. There was possibly a short initial period of fairly steep slope: but apart from this there was a period of low but steadily increasing slope, succeeded by a stage of maximum slope. Finally, there was a stage in which the slope again lessened. The transitions between these phases were gradual and the apparent change from an obvious curve to a straight line as the overall speed of the disinfection was raised was due to a gradual lessening of the differences between the slopes in the several phases. In addition, in fast reactions it becomes difficult to obtain experimental data relating to the first two of these phases because they are so quickly completed, and the mortality may have approached 90 per cent before the first sample can be taken. Clearly, in such circumstances the results cannot justifiably be used to fix the earlier parts of the disinfection curve.

At present comprehensive data have been published for one system only, namely *E. coli* and phenol, but it is known that the action of *o*-cresol on *E. coli* follows a similar pattern (Jordan, Jacobs and Penry, unpublished data). However, the establishment of disinfection curves calls for elaborate and time consuming experiments, and for that reason the technique is not suitable for the purpose of discovering how the behaviour of a disinfectant is affected by the conditions in the environment. Instead, it is customary to lighten the task by determining only "end points", that is, the times for apparently complete destruction of a population, which must therefore be of the same size in all experiments. Detailed discussion of the methods used, which are well known, would occupy too much space and seems unnecessary, and it is sufficient to point out that the end points correspond to the times required for the viable count to be reduced to some low but not in general accurately defined level of survivors. However, the mathematical treatment of end point data devised by Mather[8] yields a mean single survivor time, which is an accurately defined level of survivors.

Use of these methods has revealed that the rate of action of disinfectants may be greatly affected not only by concentration and temperature

but also by such conditions as the pH, the concentration and kind of the cations present, whether there is particulate matter in the environment and, most importantly, the kind and amount of organic matter present. It may be added that the way in which a bactericidal chemical is formulated may greatly alter its activity, but this aspect cannot be discussed here and it is sufficient to remark that the factors mentioned above will affect the rate of action of a chemical, whether formulated or not. It is also important to remember that the results will not be the same for any one chemical irrespective of the kind of organism used in the tests, and in view of all this it is evident that no useful assessment of the way in which a disinfectant will behave under the wide range of conditions likely to be encountered in practice can be obtained from the results of a single laboratory test. There is no alternative but to study the action of the disinfectant under a wide variety of conditions, and to make practical trials as well.

Useful as the end point methods are, it is nevertheless true that they yield only a fraction of the information about the course of a disinfection which is provided by the full disinfection curve, and in some circumstances a proper understanding of what has been happening in a culture exposed to a disinfectant cannot be reached unless the course of the reaction is followed in detail. Organic matter in the environment of bacteria is usually considered to have a protective action. It may react with the disinfectant and so reduce its concentration, or it may displace disinfectant from the cell surface, or may form a protective film. But it may have a further action and by serving as a source of nutrients and of energy assist the cell to maintain its structure and repair damage. Obviously this type of action could only be of significance if the other conditions were such that metabolism would be possible for some of the cells and for this to be so the concentration of the disinfectant would have to be small. This situation arose in experiments designed to determine the threshold concentration for phenol acting on standard cultures of *E. coli* at 35°. As will be seen from the results described below the value obtained was unexpectedly small, but because the full disinfection curves were determined support was afforded for a possible explanation.

The Predicted Value of the Threshold Concentration for Phenol Acting at 35° on Standard Cultures of E. coli

In the series of experiments carried out at 35° by Jordan and Jacobs[7] data were obtained showing the relation between percentage mortality and time over the phenol concentration range 3·48–8·00 g./l. From these the 99·999999 per cent mortality times (virtual sterilisation times) were calculated[9] and used to determine the concentration exponent for phenol, employing the linear relationship between log virtual sterilisation time and log phenol concentration.

The value obtained, using the full range of phenol concentrations, was 5·85 ± 0·19, and this agrees well with the value of 5·7 obtained by Tilley[10]. However, the virtual sterilisation time for 3·48 g./l. appeared rather too large, and when the concentration exponent was recalculated omitting

that result its value was reduced to $5{\cdot}66 \pm 0{\cdot}14$. Though this was not a significant reduction, the effect was to make the experimental value of the virtual sterilisation time for 3·48 g./l. even more divergent from the predicted value than it had been before. In fact it was now rather more than 30 per cent too high. This was not considered to be a reason for rejecting the lower value of the concentration exponent, but rather as an indication that 3·48 g./l. was close to the threshold concentration. At that point sterilisation takes an infinitely long time, so that the logarithm of the virtual sterilisation time must become infinitely large at a finite value of the logarithm of the phenol concentration. In other words, the graph of log virtual sterilisation time against log phenol concentration must steepen sharply as the threshold concentration is approached and the experimental values should be increasingly greater than those predicted by extrapolating the straight line which closely fits the data for higher concentrations. These relationships are illustrated in Figure 1, where line A is the straight line which best fits the data from 3·76 to 8·00 g./l. and line B is the hypothetical increasingly steep portion which should become asymptotic to a vertical line drawn through the logarithm of the threshold concentration.

The concept of the threshold concentration was subsequently developed further by Jordan and Jacobs[11], employing data obtained at different temperatures and a mathematical treatment which need not be described here, and it finally appeared likely that the value was close to 3·00 g./l. It was then decided to attempt to verify this prediction experimentally, using the same strain of *E. coli* as before, and a preliminary account will now be given of this work, which was carried out by Stone, Jacobs and Jordan.

Experimental Technique

This has been fully described by Jordan and Jacobs[1], but it is necessary to describe it briefly here because of its special features.

A standard culture of *E. coli* is developed in about 1,500 ml. of pH 7·0 phosphate buffer solution containing 1·0 g. of Difco dehydrated nutrient broth in a 5 litre round bottomed Pyrex flask held in a thermostatically-controlled water bath and agitated by means of a stream of sterile air. The dilute broth is inoculated with 300–400 viable *E. coli* cells per ml. and at the same time an automatically-operated syringe, which delivers small constant measured volumes of a solution of Difco dehydrated broth (6 g./l.) at 100 second intervals to the culture, is started. The rate of broth addition is 15·2 mg./hour. In these circumstances the bacterial population rises in a few hours to *c.* 330×10^6 viable cells per ml. and thereafter remains constant, being controlled by the rate of the continuing addition of nutrient broth. However, the total population, determined by culture turbidity, continues to rise, so that the culture consists of slowly dividing cells. After 40 hours from the inoculation the disinfection experiments are started by adding the required amount of 5 per cent phenol solution dropwise, the addition occupying 5 minutes. Thereafter samples are taken at intervals as required and the survivors counted by the

dilution plate colony count technique, with, usually, ten replicate plates per dilution.

For the attempt to determine the threshold concentration the technique was modified slightly. The initial population was *c.* 250 × 10^6 viable cells per ml. only, because it was realised that the experiments would in some instances be very long drawn out. This was achieved by reducing the initial broth addition from 1·0 g. to 0·5 g., though the regular addition

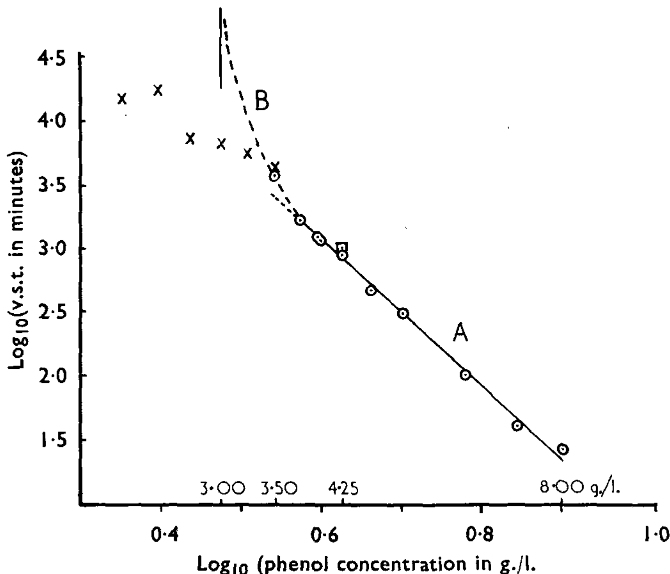

FIG. 1. The relationship between the logarithm of the virtual sterilisation time (v.s.t.) at 35° and the logarithm of the phenol concentration, for standard cultures of *E. coli.*

Circles, original data; square, newly obtained value, original conditions; crosses, newly obtained values, slightly modified conditions. The continuous line A is that which best fits the original data, its slope being 5·66 ± 0·14. The broken line B is the hypothetical continuation of line A and is asymptotic to an ordinate at the logarithm of the originally deduced threshold concentration, 3·00 g./l.

was at the same rate as before. Even so, some of the experiments lasted for over 10 days, and it was obviously necessary to ensure that the phenol concentration was not materially reduced in these long periods through losses in the air stream. Estimates of the actual loss were therefore made and the amounts made good by adding each day the required amount of 2 per cent phenol solution. The concentrations present in the cultures were checked from time to time by means of direct estimations and in no experiment was the actual concentration more than 3–4 per cent below the desired value at any time.

The concentrations used ranged from 2·0 to 3·50 g./l. in steps of 0·25 g./l. Whenever it appeared desirable an experiment was repeated, but the results were always substantially the same. In addition, the general level of resistance of the culture under the earlier experimental conditions was checked in an experiment at 4·25 g./l.

Results and Discussion

It is not possible here to present all the results in detail or to discuss all their features. This it is hoped to do in a later publication, but here only the salient features will be mentioned.

It is important to note that the organism used was still behaving substantially as it had in the earlier experiments, as judged by the virtual sterilisation times at 4·25 and 3·50 g./l. As shown in Figure 1, the logarithms of these were very close to the values expected from the earlier results, though actually the cultures were rather more resistant than before, the numerical values of the virtual sterilisation times both being about 16 per cent larger than expected. Despite this, however, the cultures were notably more sensitive than expected at concentrations below 3·50 g./l., and so far from the threshold concentration being close to

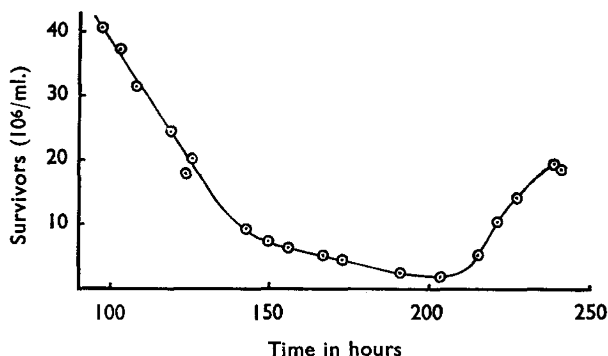

Fig. 2. The changes in viable cell count in the later stages of an experiment in which a standard culture of *E. coli* was exposed at 35° to phenol at 2·00 g./l., showing the ultimate occurrence of growth.

3·00 g./l. complete destruction was obtained down to 2·25 g./l. (Fig. 1). But not only was the threshold concentration lower than expected; the pattern of behaviour of the cultures had altered and instead of the expected steady rise in slope of the graph of log virtual sterilisation time against log concentration there was an increasingly wide divergence until suddenly, at 2·00 g./l., sterilisation was not complete. In fact, at that concentration, multiplication occurred after the viable count had fallen to about $1{\cdot}75 \times 10^6$/ ml., i.e., 0·83 per cent of its initial value (Fig. 2), and a further point of interest is that the virtual sterilisation time for 2·25 g./l. was not larger but smaller than that for 2·50 g./l. (Fig. 1).

It is evident that in these cultures some disturbing factor was at work, and consideration of certain features of the disinfection curves leads to the conclusion that the experimental conditions had permitted growth to occur, with accompanying alterations in the sensitivities of varying proportions of the cells in the cultures.

It is evident that some cells of a culture can grow in the presence of a concentration of phenol which is lethal to most cells, as shown by the

later part of the survivor curve at 2·00 g./l. (Fig. 2). The fact that growth occurred indicates that available food was present, and there is no doubt that the rate of food addition was for a long time in excess of the amount which could be utilised by the falling viable population. This was below 10×10^6/ml. for at least 60 hours before the growth commenced, and as in that time over 0·9 g. of food was added there should have been ample to support quite a large population. But the increase in viable count was not extensive and there are also signs that it had reached its limit at about 20×10^6 cells per ml. It is, therefore, perhaps remarkable that the growth was so restricted. However, a possible explanation is suggested by the

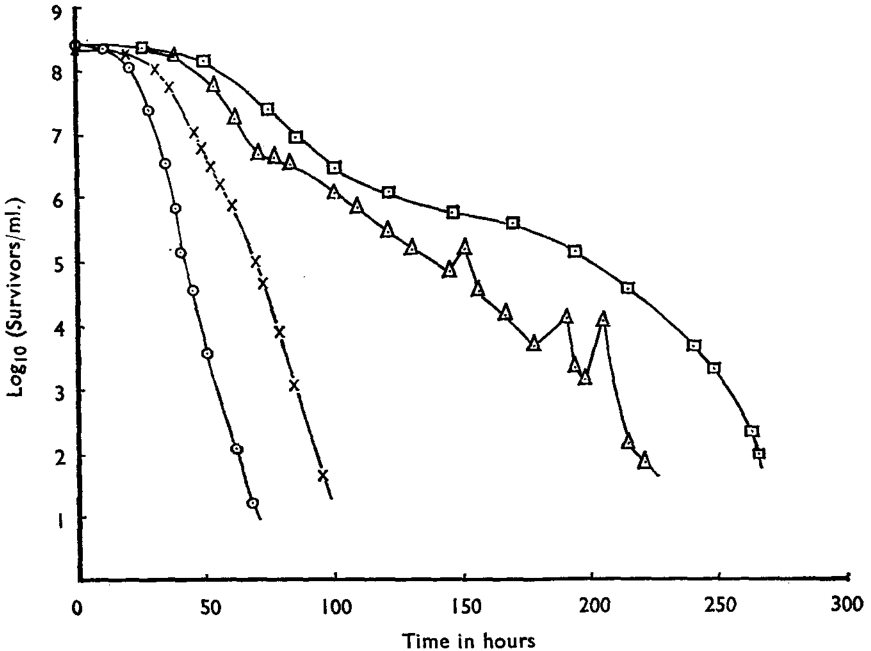

FIG. 3. The relationships between the logarithm of the number of survivors and time in standard cultures of *E. coli* exposed at 35° to phenol. The concentrations used, in g./l., were: 3·50 (circles), 3·00 (crosses), 2·50 (squares) and 2·25 (triangles).

fact that the increase in turbidity which occurred at this stage was much larger than the observed rise in viable cells could by itself have produced. The turbidity rose from 20 per cent below to 20 per cent above the initial value, and as the viable cell increase could have accounted for about one-sixth only of this, it seems clear that many more cells must have been formed, but had been killed. Thus rapid death must have accompanied the multiplication stage, and it is possible that had the experiment been continued the viable count might have dropped again and even ultimately reached zero. The possibility of this is indicated by the viable counts in the later stages of the experiment at 2·25 g./l., where marked sudden fluctuations occurred before the count finally reached zero, as shown in

Figure 3. This phenomenon has not been observed before in the now extensive series of experiments on these standard cultures. These particular fluctuations did not coincide with marked turbidity changes: but this is not surprising since they occurred at a time when the viable population was of the order of thousands per ml. only.

Since it appears that rapid death may accompany an increase in viable count in these slow disinfections, it seems logical to enquire whether multiplication may not also have been proceeding during the phase of continual decline in count, as it does in the decline phase of a normal culture[12]. Evidence is provided by abnormalities in the shapes of the graphs of log per cent survivors against time for 2·50 and 2·25 g./l. As shown in Figure 3, whereas the graphs for 3·50 and 3·00 g./l. were quite similar to the general pattern described above, at 2·50 and 2·25 g./l. there was a strongly marked intermediate period of high slope followed by a long period of low slope before a further steepening occurred. This change could have been due to the production of new cells, food and energy being available because of the lessened demand on the food being supplied and the phenol concentration not being too high to prevent the growth of all cells. It is also conceivable that even if cells were not multiplying they were succeeding in repairing the damage caused by the phenol and so increasing their survival time. However, the effect of cell repair and multiplication, it might be supposed, would be to decrease the overall rate of action of a given phenol concentration, whereas the virtual sterilisation times obtained were in fact much shorter than expected. Also, there are other indications of an abnormally increased sensitivity of the cells in these cultures in certain circumstances, namely the marked relatively rapid cell death in the intermediate stages at 2·50 and 2·25 g./l. There is also the fact that 2·25 g./l. was not less but more effective than 2·50 g./l. (Fig. 3).

Now while no fully comprehensive explanation of the experimental results can be offered, much of the behaviour of these cultures, in which the conditions were not only complex but changing in different ways in different circumstances, can be accounted for with the aid of two concepts: first, that the inherent ability of a cell to withstand damage varies according to the stage it has reached in the division cycle, which implies that at a particular stage resistance is minimal; and second, that after fission in the presence of phenol the daughter cells are more susceptible than the parent. The first concept, which has already been put forward by Hinshelwood[13] to explain the abnormal protraction of the final stages of decline in supposedly uniform populations exposed to lethal agents, is difficult to reconcile with the occurrence of an almost straight line relationship between log survivors and time, as pointed out by Eddy[14]; but this difficulty does not exist where that relationship does not hold, as in the present work. It must be added that for the present purposes the level of resistance at the minimum is considered to differ among the cells of a culture, and indeed it may be supposed that some cells may always display a higher level of resistance than others at corresponding stages of the division cycle.

By level of resistance is meant the period of exposure the cell can tolerate in the absence of an external supply of nutrients and still be viable when transferred to a nutrient medium. If nutrients are available during exposure repair may be possible and survival prolonged, and if the supply is sufficiently large and the phenol concentration sufficiently low growth may occur and the cell will proceed through the division cycle. But according to the initial position of the cell in that cycle it may become either more or less resistant. If the latter, death may occur sooner than it might otherwise have done, while cells whose resistance was increased may succeed in dividing, only to be brought in their turn towards the condition of minimal resistance and to their death. In this way an accumulation of food may not only accelerate death but induce multiplication followed by accelerated death. However, if daughter cells are always closely similar to their parents in resistance level some cells of high resistance would be expected to survive in low phenol concentrations and continue to multiply till a new equilibrium with the food supply was established. But that did not occur in the present experiments and there were apparently spurts of multiplication followed or accompanied by rapid death. To account for these findings a progressive increase in inherent sensitivity following division of cells in the presence of phenol has been postulated. In this connection it may be recalled that Van Eseltine and Rahn[15] observed transient waves of growth in broth cultures of *E. coli* containing low concentrations of phenol.

This consideration of the results of experiments conducted on cultures in which the conditions were changing in a complex way has necessarily been superficial, but the ideas introduced do make it possible to imagine how a lower concentration could be more effective than a higher one, either at all stages of a disinfection process or at certain stages only, in terms of an interaction between the effects of phenol and of nutrients on the metabolism of individual cells at different stages of the division cycle. The further idea of a general increase in sensitivity of cells following division in the presence of phenol is not an unreasonable one, for such cells might easily be less well endowed with reserves than normal cells.

In conclusion, it may be remarked that whereas organic matter is known to protect bacteria against disinfectants the experiments described above have shown that in certain circumstances, for example, at disinfectant concentrations approaching the threshold value, utilisable organic matter may make a culture as a whole more sensitive. The experiments failed to fix the threshold concentration for phenol acting on *E. coli* at 35° because the conditions changed too greatly during the disinfections. It will probably be impossible to adjust the food supply exactly to the changing demands as cells are killed and so to make possible an accurate determination of the true threshold concentration.

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