STUDIES ON BACTERIAL POPULATIONS IN SOLUTIONS OF PHENOLS

Part II. The Influence of Cell-exudate upon the Shape of the Survivor-time curve

By H. S. Bean and V. Walters*

From Chelsea School of Pharmacy, Chelsea College of Science and Technology, Manresa Road, London, S.W.3

Received April 7, 1961

The shape of time-survivor curves for *E. coli* suspended in aqueous solutions of benzylchlorophenol depends on the concentration of the bactericide. In some concentrations the last survivors exhibit unexpected multiplication. The death of the organisms is accompanied by the release of cell-constituents having an ultra-violet absorption maximum at 260 mμ. Growth occurred when *E. coli* exudate was added to aqueous suspensions of *E. coli* whereas in its absence the organisms died. The numbers of viable organisms depended upon the concentration of the added cell exudate. The fate of the last survivors of a suspension of *E. coli* in benzylchlorophenol solution was controlled by re-suspending them in components of the reaction mixture. Removal of cell-exudate produced rapid death and the removal of bactericide led to multiplication of the organisms. It is concluded that cell exudate constituted the nutrient substrate for multiplication of last survivors in an initially bactericidal system. The theories of disinfection and the influence of cell exudate upon the shape of time-survivor curves are discussed.

Washed *Escherichia coli* suspended in a solution containing 50 μg./ml. benzylchlorophenol in 0·01M phosphate buffer (pH 7·0) unexpectedly multiplied after about 7 hr., by which time the survivors had diminished to less than 0·01 per cent of the initial number (Bean and Walters, 1955). During the ensuing 100 hr. or more, a 50 to 500-fold increase in the viable count occurred, and in replicate experiments the maximal counts reached 0·5 to 1·0 per cent of the initial count. The system contained no nutrient material in the accepted sense. Nevertheless, the marked increase in the viable count indicated the existence of a source of nutrient. We suggested that the observed multiplication could be explained by the few surviving organisms utilising water-soluble constituents released from dead organisms. But it could have been due to the utilisation of the phosphate buffer which contained Na^+ and K^+ mono- and di-hydrogen phosphates and, possibly, traces of organic impurities. Many workers (Allan, Pasley and Pierce (1952); Bigger (1937); Bigger and Nelson (1941, 1943); Butterfield (1929); Garvie (1955); Savage and Wood (1917)) have shown that bacteria can multiply in the presence of only traces of nutrient materials.

The present paper reports further experiments to test the hypothesis that *E. coli* can utilise the exudate from cells of a culture and to ascertain whether multiplication of the last survivors in a suspension can occur in various concentrations of benzylchlorophenol.

* Present Address: Nigerian College of Technology, Ibadan, Nigeria.

Experimental

The test organism (*Escherichia coli* Type 1) and experimental methods were as described previously (Bean and Walters, 1955). The improved capillary-dropping pipette (Cook and Yousef, 1953; Cook 1954) was used for sampling the bacterial suspensions and the viable counts were made by the standard roll-tube technique (Berry and Michaels, 1947; Wilson, 1922; Withell, 1938).

The Viability of Untreated E. coli *in Solutions of Cell Exudate*

A sterile solution of cell exudate (optical density $d_{1\,cm.}$ at 260 mμ = 1·2) was prepared by heating thrice-washed *E. coli* in distilled water at 100°

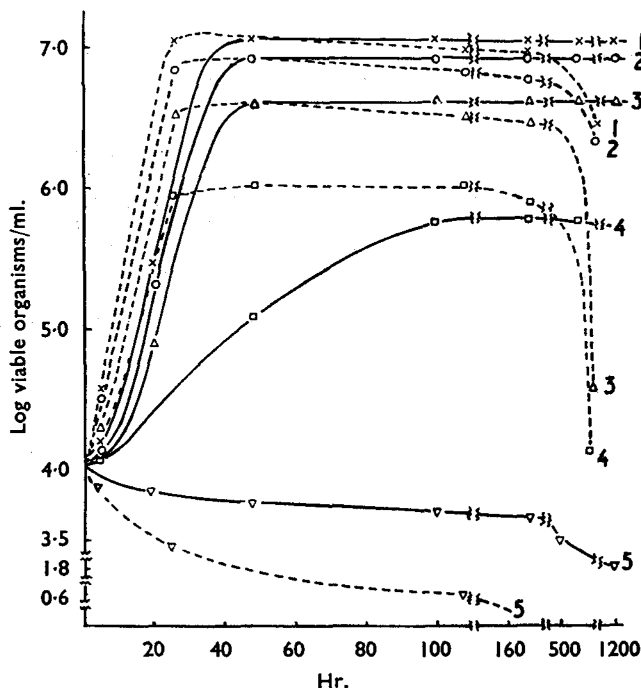

Fig. 1. Effect of concentration of cell exudate solution upon the growth of *E. coli* at 20° C (solid lines) and at 37° (broken lines).
Curves 1–4 represent cell exudate solution having $d_{1cm.}$ at 260 mμ = 1·2, 0·9, 0·6 and 0·3 respectively, curve 5 = distilled water (reference curve).

for 5 min., removing the cells by centrifugation and filtering the supernatant through a sterile 5-on-3 sintered glass filter. The filtrate was diluted with sterile distilled water to give three further solutions containing respectively 75, 50 and 25 per cent of the original exudate solution. To 60 ml. quantities of each of the four solutions and to distilled water as a control, one drop (0·016 ml.) of a diluted washed aqueous suspension of *E. coli* was added. The inoculated solutions were divided into two equal portions, maintained at 20° and 37° respectively, and viable counts were made at intervals.

When suspended in distilled water the organisms died slowly at 20° and much more rapidly at 37° (Fig. 1). In solutions of cell exudate they showed typical growth curves. Each phase of the growth cycle was longer at 20° than at 37°, the optimal temperature for growth (Wilson and Miles, 1955). The increase of number of organisms at the end of the log phase of growth in the respective exudate solutions was about

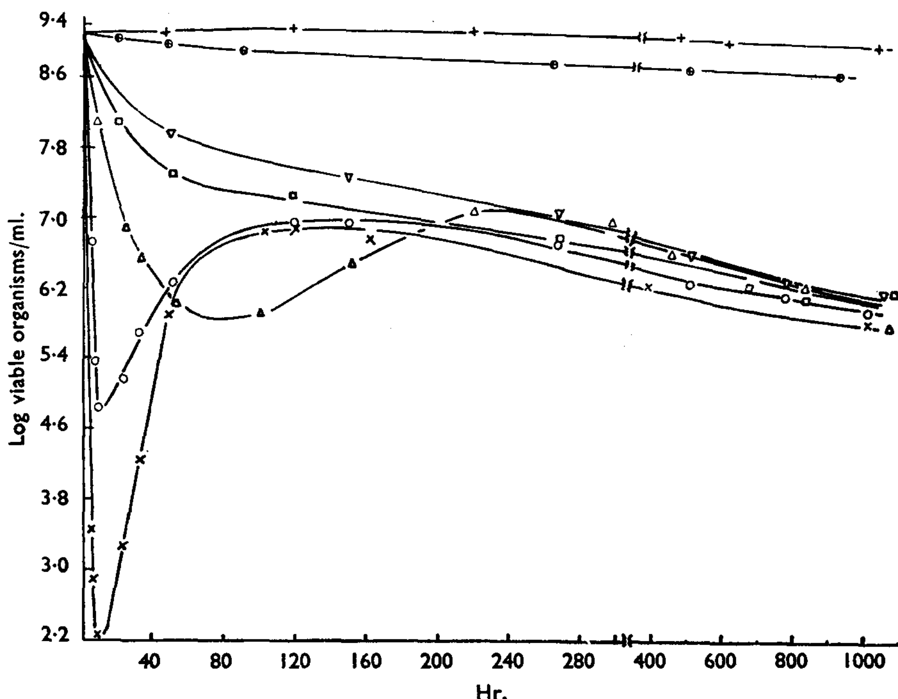

FIG. 2. Time-log survivor curves for *E. coli* in
+——+ cell exudate ($d_{1cm.}$ at 260 mμ = 1·25)
⊕——⊕ distilled water
▽——▽ benzylchlorophenol 20 μg./ml.
□——□ " 25 "
△——△ " 27·5 "
○——○ " 33 "
×——× " 50 "

1,000 fold. It was related to the concentration of the cell exudate solution and was approximately the same at both incubation temperatures.

The optical density at 260 mμ of the cell-free solutions showed that multiplication of the organisms was accompanied by a very small decrease in the concentration of cell exudate. On the assumption that the wet weight of 10 $\times$ 10^6 organisms was 0·01 mg. (Oginsky and Umbreit, 1954) and that each bacterium was composed of 80 per cent water (Wilson and Miles, 1955), then 10 $\times$ 10^6 *E. coli* would have had a dry weight of only 2 μg. Hence, the total mass of organic material synthesised was very small and accounted for the small decrease in cell exudate concentration.

Dead and dying cells would also have contributed some exudate to the solution during the period of the experiment.

The viability of E. coli *in Aqueous Solutions of Benzylchlorophenol*

Phosphate buffer, or impurities therein has been reported to promote the growth of *E. coli* (Garvie, 1955; Chambers, Tabac and Kabler, 1957). To determine whether multiplication of the survivors in solutions of benzylchlorophenol occurs in the absence of the phosphate buffer used

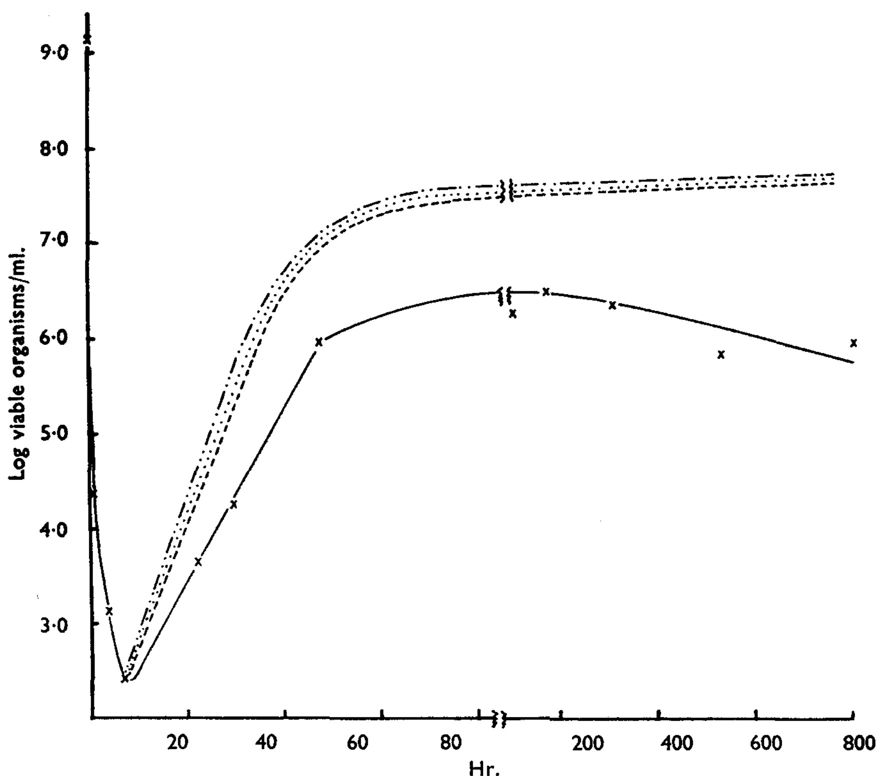

FIG. 3. Time-log survivor curves for *E. coli* in 50 μg./ml. benzylchlorophenol (×——×) and for organisms washed after 7½ hr. and re-suspended in solutions of cell exudate (broken lines).

--·--·· $d_{1cm.}$ at 260 mμ = 0·46
········ ,, ,, = 0·20
or ------ distilled water.

in the earlier experiments, the viability of *E. coli* was determined in unbuffered solution of the phenol at 50 μg./ml.

Viable counts were made at intervals on *E. coli* in solutions containing respectively 20, 25, 27·5, 33·3, 50 and 100 μg./ml. of the phenol. The results, with the exception of those for the 100 μg./ml. solution, in which all the organisms died within 5 min., are presented in Figs. 2 and 3. In Fig. 2, the log survivor-time curves of *E. coli* in distilled water and in cell exudate solution ($d_{1\,cm.}$ = 1·25 at 260 mμ) are also shown for comparison.

The shape of the log survivor-time curve varied markedly with the concentration of the phenol. In the weakest solutions of 20 and 25 μg./ml., there was a gradual and continual decrease in the viable count which produced shallow sigmoidal curves. In the 27·5 μg./ml. solution, a rapid initial decrease in the viable count was followed by an equally rapid increase, and in the 33·3 and 50 μg./ml. solutions an extremely rapid decrease was followed by an almost equally rapid increase.

These results show conclusively that multiplication of the last survivors in solutions of benzylchlorophenol does occur in the absence of phosphate buffer and in concentrations of the phenol other than 50 μg./ml. A comparable multiplication of the last survivors in a solution of a chemical bactericide has not, to the authors' knowledge, hitherto been recorded, although Jacobs (1960) has recently observed multiplication of the last survivors of a suspension of *E. coli* in dilute broth containing phenol.

The viable counts during the period 250–1,100 hr. in all solutions containing benzylchlorophenol were within similar limits, indicating that common factors were limiting and controlling the multiplication of the last survivors. These factors are likely to be the same as those which produce the stationary phase of growth in liquid cultures, namely exhaustion of food supply, accumulation of toxic products or oxygen starvation.

Influence of Components of Reaction Mixture on the Viability of Last survivors

To justify the conclusion that the multiplication of the last survivors depended on the presence of cell exudate *E. coli* were treated with benzylchlorophenol, washed, and re-suspended either in sterile distilled water, cell exudate or solutions of the phenol, or in mixtures of the two latter.

Benzylchlorophenol solution 50 μg./ml. was selected as the bactericide because previous experiments had indicated that the survivors after 7·5 hr. could be as few as 1 in 10^7; the system would then contain an appreciable quantity of cell exudate. After 7·5 hr. 50 ml. quantities of the reaction mixture were centrifuged at 8,500 *g* for 3 min., the supernatant fluid removed, the organisms washed and re-suspended in the appropriate sterile fluid to produce approximately the same number of viable organisms as in the reaction mixture.

Viability after re-suspension in water and in exudate solutions. Two cell exudate solutions were used. One ($d_{1\,cm.}$ at 260 mμ = 0·20) was approximately equivalent in concentration to that produced by *E. coli* during 7·5 hr. in 50 μg./ml. of benzylchlorophenol and the other more than twice that concentration of exudate. After 7·5 hr. the reaction mixture contained only 252 viable cells/ml. (i.e. 2 in 10^7 of the original 2×10^9) survivors; the numbers in the reconstituted suspension in cell exudate solutions ($d_{1\,cm.}$ at 260 mμ = 0·46 and 0·20) and distilled water were 213, 179 and 218/ml. respectively. The viable count of the reconstituted suspensions increased more rapidly than that of the reaction mixture (Fig. 3). After 150 hr. about eleven times as many viable organisms were in the reconstituted suspensions as in the reaction mixture,

and at this time the count in the reaction mixture reached a peak indicating that the rate of multiplication in the reaction mixture was influenced by the residual concentration of the bactericide. At the end of the experimental period of nearly 800 hr. the reconstituted suspensions contained about 45 times as many viable organisms as did the control reaction mixtures. The rate of multiplication immediately after resuspension was greater in the cell exudate solutions than in the distilled water. Later, the differences between the rates diminished.

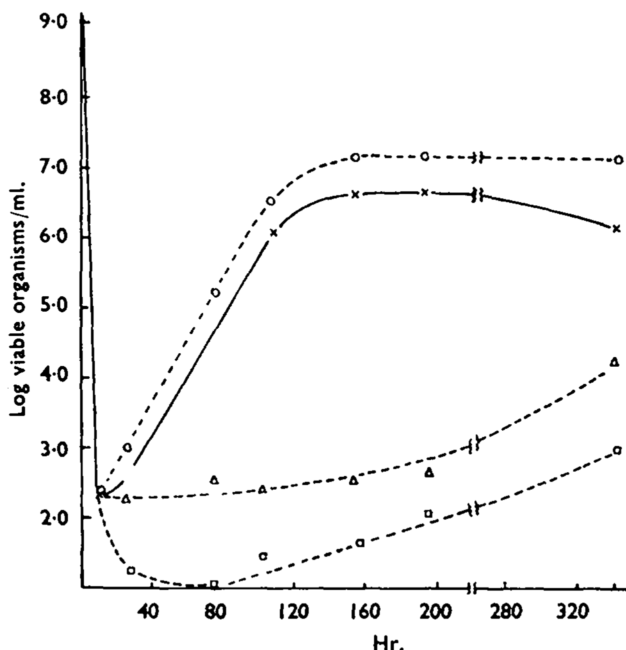

FIG. 4. Time-log survivor curves for *E. coli* in 50 μg./ml. benzylchlorophenol (× —— ×) and for organisms washed after 7½ hr. and re-suspended.

○——○ re-suspended in 20 μg./ml. benzylchlorophenol
△——△ " 30·75 " "
□——□ " 40 " "

Viability after re-suspension in solutions of benzylchlorophenol without added cell exudate. When *E. coli* had been suspended in benzylchlorophenol 50 mg./ml. for 7·5 hr., the residual bactericide in the supernatant was found to be 30·75 μg./ml. (Walters, 1959). To determine the influence of the phenol in the re-suspending medium, treated organisms were re-suspended in solutions containing 20, 30·75 and 40 μg./ml. of the phenol but no exudate. The increase in concentration retarded the increase in the viable count (Fig. 4). In the 20 μg./ml. re-suspension, multiplication was at a slightly greater rate than in the reaction mixtures. The 30·75 μg./ml. solution (i.e. the reaction mixture minus exudate), was bacteriostatic but after a considerable lag, multiplication occurred. The

40 μg./ml. solution was bactericidal. The survivors decreased to approximately 1 in 2×10^8 of the initial viable count of the reaction mixture, but ultimately multiplied as exudate became available from dying cells.

Viability after re-suspension in solutions of benzylchlorophenol with added cell exudate. The influence of different concentrations of cell exudate on multiplication was assessed by re-suspending treated organisms in benzylchlorophenol solution 30·75 μg./ml. without and with cell exudate

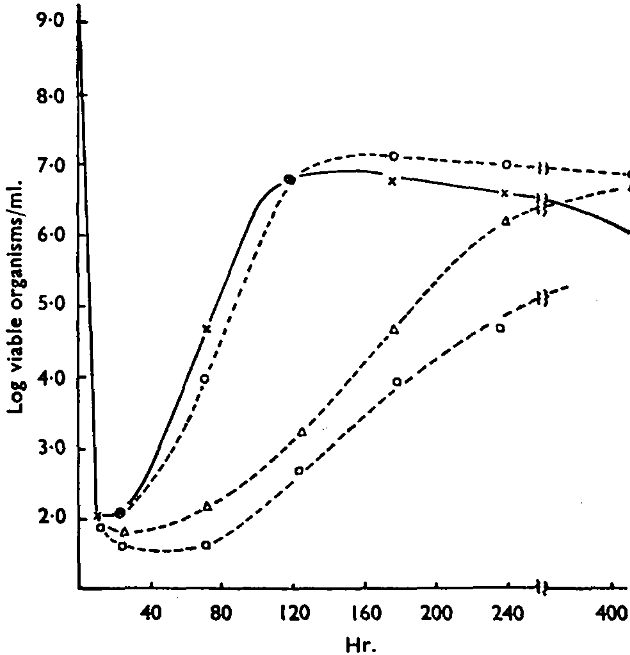

FIG. 5. Time-log survivor curves for *E. coli* in 50 μg./ml. benzylchlorophenol (×——×) and for organisms washed after $7\frac{1}{2}$ hr. and re-suspended.

○ — — ○	re-suspended in 30·75 μg./ml. benzylchlorophenol + exudate ($d_{1cm.}$ at 260 mμ = 0·70)
△ — — △	re-suspended in 30·75 μg./ml. benzylchlorophenol + exudate ($d_{1cm.}$ at 260 mμ = 0·16)
□ — — □	re-suspended in 30·75 μg./ml. benzylchlorophenol. (No exudate added.)

added at the concentration estimated in the reaction mixture after 7·5 hr. ($d_{1\,cm.}$ at 260 mμ = 0·16) and at approximately $4\frac{1}{2}$ times that concentration of exudate ($d_{1\,cm.}$ at 260 mμ = 0·70). When the re-suspension contained no cell exudate there was a further fall in the viable count, followed by a period of no-change in the count and ultimately by multiplication. The effect on the count of adding no exudate was, in fact, similar to that of increasing the supernatant concentration of the phenol in the reaction mixture, after 7·5 hr. (Figs. 4 and 5).

Re-suspending the treated organisms in a solution of the phenol and exudate at concentrations found in the control reaction mixture after

7·5 hr., caused a shorter lag than in the system containing no exudate (Fig. 5) but a longer lag and slower rate of multiplication than in the control. Increasing the exudate concentration 4·5-fold produced a growth cycle almost coincident with that of the control reaction mixture.

Re-suspension of E. coli *after Exposure to Benzylchlorophenol* 27·5 *and* 33·3 *μg./ml.*

Whether multiplication could be induced at different stages in the bactericidal reaction, was challenged with more dilute solutions containing 27·5 and 33·3 μg./ml. of benzylchlorophenol in which the reaction velocity

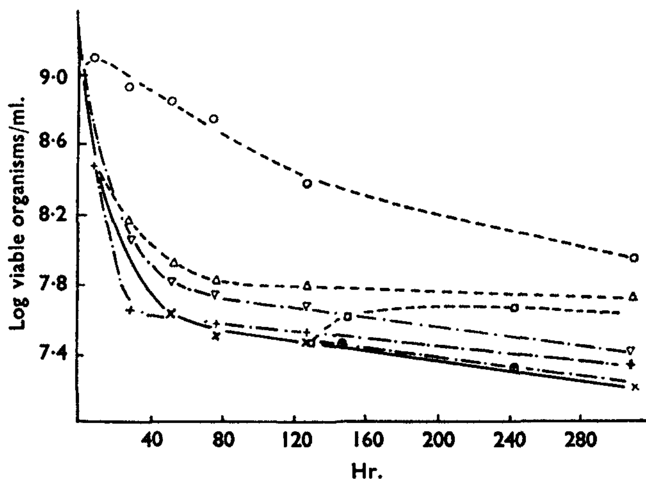

Fig. 6. Time-log survivor curves for *E. coli* in 27·5 μg./ml. benzylchorophenol (× —— ×) and for organisms washed after 1, 7 and 126 hr. and re-suspended.

○ — — ○ after 1 hr. re-suspended in cell exudate (d_{1cm} at 260 mμ = 0·15)
△ — — △ after 7 hr. re-suspended in cell exudate (d_{1cm} at 260 mμ = 0·17)
□ — — □ after 126 hr. re-suspended in cell exudate (d_{1cm} at 260 mμ = 0·38)
▽ — — ▽ after 1 hr. re-suspended in 20·5 μg./ml. benzylchlorophenol. No exudate.
+ — — + after 7 hr. re-suspended in 20·5 μg./ml. benzylchlorophenol. No exudate.
⊕ — — ⊕ after 126 hr. re-suspended in 20·5 μg./ml. benzylchlorophenol. No exudate.

was slower. The reactions were allowed to proceed until the survivors were estimated to approximate to 50, 20 and 0·1 per cent of the original population. Aliquots were therefore taken after the reaction had been in progress for 1, 7 and 126 hr. in the 27·5 μg./ml. solution and after 1 and 7 hr. in the 33·3 μg./ml. solution. The organisms were spun, washed and re-suspended, either in cell exudate or the phenol solution at the concentrations estimated in the supernatant fluid after the specified reaction periods. Multiplication was observed when the organisms were re-suspended in solution of cell exudate (Figs. 6 and 7). In both reaction mixtures the longer the duration of the reaction before re-suspension, the higher the mortality and the greater the rate of multiplication after re-suspension. This was expected since the higher mortalities produced greater amounts of cell exudate in the supernatant.

In the suspension of *E. coli* in solution of the phenol at 27·5 μg./ml. (Fig. 6) the mortality did not exceed 99·9 per cent and no multiplication was observed in the reaction mixture. It appeared that the resistance of the organisms had increased in the many months during which the experiments lasted since a mortality of 99·93 per cent followed by multiplication was previously observed with a solution of this concentration. The difference in behaviour of the organisms in the two experiments with 27·5 μg./ml. of the phenol emphasises that one factor determining whether or not multiplication occurs is the concentration of cell exudate in the supernatant fluid.

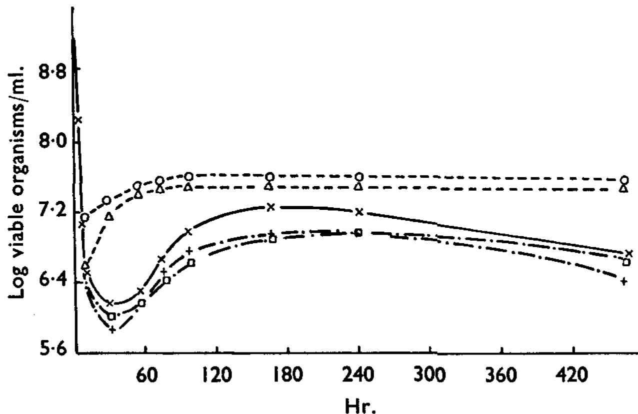

FIG. 7. Time-log survivor curves for *E. coli* in 33·3 μg./ml. benzylchlorophenol (× —— ×) and for organisms washed after 1 and 7 hr. and re-suspended.

○ — — ○ after 1 hr. re-suspended in cell exudate ($d_{1cm.}$ at 260 mμ = 0·16)
△ — — △ after 7 hr. re-suspended in cell exudate ($d_{1cm.}$ at 260 mμ = 0·18)
+ — — + after 1 hr. re-suspended in benzylchlorophenol 25 μg./ml. No exudate
□ — — □ after 7 hr. re-suspended in benzylchlorophenol 25 μg./ml. No exudate

The time-survivor curves for the organisms re-suspended in solutions of benzylchlorophenol followed the same general pattern as the respective reaction mixture control curves. With the exception of the 1 and 7 hr. re-suspensions derived from the reaction mixture containing 27·5 μg./ml. of the phenol in which the mortality had not reached a low level, the viable counts for the re-suspensions were less than those for the control suspension. This indicated that in the normal reaction mixture the viable count, in particular at high mortality levels, was influenced and increased by the presence of cell exudate.

An attempt was made by direct microscopical count and by turbidity measurements to verify the increase in the viable count. The results were inconclusive. This is perhaps not surprising since an increase in the viable count from 2 to 3 × 10^2 per ml. to as many as 40 × 10^6 *E. coli* per ml. which were present in some of the cell exudate re-suspensions, represented a total increase of only 3 per cent of the initial viable count whereas the accuracy of microscopical counts is ±10 per cent (Wilson and Miles, 1955).

Discussion

The log time-survivor curves for the viability of *E. coli* in solutions of benzylchlorophenol varied from shallow sigmoidal curves to those showing an extremely rapid logarithmic decrease in the viable count followed by a rapid increase and finally a slow decline. The pattern of the increase in the count followed that represented by a typical bacterial growth curve and was similar to that of the multiplication of *E. coli* in cell exudate solution. It is concluded that the increase in the viable count was due to the multiplication of the last survivors and that the necessary nutrient was obtained from cell constituents released into the suspending fluid from dead and dying cells.

The following appear to be essential requirements for multiplication in a bactericidal system. Organisms which, upon their death, release cell constituents on which organisms of the same strain can multiply. A large inoculum to provide an adequate amount of cell exudate. A concentration of bactericide which reduces the viable population to a very low number (>99·9 per cent mortality) without causing 100 per cent mortality, and thus producing a sufficient concentration of cell exudate for the multiplication of the survivors. A bactericide which is active in dilute solution and has a high concentration exponent so that removal of the bactericide from the solution by adsorption upon the bacterial surface considerably depletes the suspending fluid of bactericidal power.

The significance of the shape of the time-survivor curve is discussed at length in the literature. It is interpreted to indicate, on the one hand, that the bactericidal reaction is similar to a unimolecular chemical reaction and, on the other, that the length of time an organism can survive when exposed to a bactericide is proportional to its resistance to the bactericide. There are many instances of poor fit to the exponential equation. There are also many instances of small but significant numbers of survivors having been found after extended exposure times. Extrapolation of the time-survivor curves would in such cases have indicated very low probabilities of any survivors and sterility would have been assumed. This is nowhere more evident than in our own experiments and indicates that completely erroneous conclusions about sterilisation times may be drawn by the extrapolation of curtailed viable count observations. The evidence presented indicates that the application of the unimolecular law to the disinfection reaction should be regarded as no more than a convenient method of treating disinfection data. Clearly, it should be applied with extreme caution. This was emphasised by Chick (1910, 1930) and more recently, by other workers (Bullock, 1956; Hinshelwood, 1951; Phillips and Warshowsky, 1958).

It is possible that both the mechanistic theory of disinfection which assumes chance interactions of a bactericide with "sensitive" regions of cells possessing similar resistance (Chick, 1908, 1910; Rahn, 1945) and the vitalistic theory which assumes a progressive destruction of vital activities in cells possessing a variation in inherent resistance (Withell, 1942; Smith, 1921, 1923; Berry and Michaels, 1947b) together provide a truer explanation than does either alone for the maintenance

or loss of viability of organisms exposed to a bactericide, and hence for the shape of time-survivor curves. In the presence of a strong bactericide the speed of the chance events leading to death would dominate and mask any small difference in resistance between the cells. This would suggest apparently logarithmic survivor curves. Under mild disinfectant conditions any variation in the resistance of individual cells would be of more significance and the tendency would be towards sigmoidal survivor curves. The prolonged survival of cells towards the end of a mild disinfection process may also be due to increased resistance the cells acquire as a result of slow adaptive adjustment to the adverse conditions.

With the *E. coli*–benzylchlorophenol system a further explanation is necessary for the shape of the time-survivor curves. Multiplication of the small number of survivors remaining towards the end of the reaction occurs as a result of the utilisation of nutrient cell constituents released from dead cells.

Multiplication in an initially bactericidal system may be confined to reaction systems of this type. It is, however, possible that the reduction in the mortality rate with time which has been frequently observed towards the end of disinfection reactions, and which has been attributed to the greater resistance of the last survivors, may, in part, be due to the presence in the suspending fluid of cell constituents. Even if the last survivors do not multiply their survival times may be much prolonged. Certainly, neither the mechanistic nor the vitalistic theory of disinfection can alone offer a complete explanation for the course of the reaction reported in this communication.

REFERENCES

Allen, L. A., Pasley, S. M. and Pierce, M. A. (1952). *J. gen. Microbiol.*, **7**, 36–43.
Bean, H. S. and Walters, V. (1955). *J. Pharm. Pharmacol.*, **7**, 661–675.
Berry, H. and Michaels, I. (1947a). *Quart. J. Pharm. Pharmacol.*, **20**, 331–347.
Berry, H. and Michaels, I. (1947b). *Ibid.*, **20**, 348–367.
Bigger, J. W. (1937). *J. Path. Bact.*, **44**, 167–211.
Bigger, J. W. and Nelson, J. H. (1941). *Ibid.*, **53**, 189–206.
Bigger, J. W. and Nelson, J. H. (1943). *Ibid.*, **55**, 321–327.
Bullock, K. (1956). *J. Pharm. Pharmacol.*, **8**, 689–708.
Butterfield, C. T. (1929). *Pub. Hlth. Rep. Wash.*, **44**, 2865–2872.
Chambers, C. W., Tabak, H. H. and Kabler, P. W. (1957). *J. Bact.*, **73**, 77–84.
Chick, H. (1908). *J. Hyg. (Camb.)*, **8**, 92–158.
Chick, H. (1910). *Ibid.*, **10**, 237–286.
Chick, H. (1930). *A System of Bacteriology in Relation to Medicine.* Vol. I, p. 179. London: H.M.S.O.
Cook, A. M. (1954). *J. Pharm. Pharmacol.*, **6**, 629–637.
Cook, A. M. and Yousef, R. T. (1953). *Ibid.*, **5**, 141–144.
Garvie, E. I. (1955). *J. Bact.*, **69**, 393–398.
Hinshelwood, C. (1951). *Nature, Lond.*, **167**, 666–669.
Jacobs, S. E. (1960). *J. Pharm. Pharmacol.*, **12**, *Suppl.* 9*T*–18*T*.
Jordan, R. C. and Jacobs, S. E. (1944). *J. Hyg. (Camb.)*, **43**, 275–289.
McQuillen, K. (1956). *Bacterial Anatomy*, pp. 127–149. 6th Sym. Soc. gen. Microbiol., Cambridge: University Press.
Mitchell, P. (1954). *J. gen. Microbiol.*, **11**, 73–82.
Oginsky, E. L. and Umbreit, W. M. (1954). *An Introduction to Bacterial Physiology.* San Francisco: Freeman.
Phillips, C. R. and Warshowsky, B. (1958). *Ann. Rev. Microbiol.*, **12**, 525–550.
Rahn, O. (1945). *Injury and Death of Bacteria by Chemical Agents.* Normandy, Missouri: Biodynamica.
Salton, M. R. (1956). *Bacterial Anatomy*, pp. 81–110. 6th Sym. Soc. gen. Microbiol., Cambridge: University Press.

Savage, W. G. and Wood, D. R. (1917). *J. Hyg. (Camb.)*, **16**, 227–239.
Smith, J. H. (1921). *Ann. appl. Biol.*, **8**, 27–50.
Smith, J. H. (1923). *Ibid.*, **10**, 335–347.
Stanier, R. Y. (1954). *Cellular Metabolism and Infections*, p. 3, New York: Academic Press.
Storck, R. L. and Wachsman, J. T. (1957). *Biochem. J.*, **66**, 19P–20P.
Walters, V. (1959). *Studies on Bacterial Populations in Solutions of Phenols.* Ph.D. thesis, University of London.
Wilson, G. S. (1922). *J. Bact.*, **7**, 405–446.
Wilson, G. S. and Miles, A. A. (1955). *Topley and Wilson's Principles of Bacteriology and Immunity.* 4th ed., London: Arnold.
Withell, E. R. (1938). *Quart. J. Pharm. Pharmacol.*, **11**, 736–757.

The paper was presented by DR. BEAN.