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The partially inhibited growth of *Escherichia coli* in the presence of some antibacterial agents

R. M. RYE AND DAVID WISEMAN

Cultures of *Escherichia coli* were partially inhibited by treatment with tetracycline, phenol, phenylmercuric acetate or cetyltrimethylammonium bromide. The effects of these agents were investigated by measuring the cell size distributions after growth had occurred in the presence of sufficient ampicillin to suppress cellular division. Tetracycline and phenol inhibited cultures by a uniform decrease in the rate of growth of all of the cells; cetyltrimethylammonium bromide completely inhibited the growth of some of the cells whilst having no effect on the remainder; phenylmercuric acetate probably affected all the cells but inhibited each individual to a different extent. The implications of these results are discussed in terms of a general growth rate equation.

THE growth rate of bacterial cultures is reduced in the presence of sub-inhibitory concentrations of antibacterial agents. Most studies of this phenomenon have been concerned with the development of techniques for microbiological assay (Kavanagh, 1963) and few attempts to determine the mechanism of growth reduction have been made. The two mechanisms by which this overall decrease in the growth rate may be achieved are (1) a uniform inhibition of all of the cells, and (2) a non-uniform inhibition of the individual cells. The second mechanism embraces both the complete inhibition of the growth of some of the cells with the remainder growing at the normal rate (Treffers, 1956) and the situation where all the cells are growing but at widely different rates. Studies of the mechanism of inhibition have previously been based on simultaneous measurements of the total and viable cell counts (Parkinson & Pickett, 1964; Garrett & Miller, 1965) but this approach is not entirely satisfactory as organisms not multiplying under the conditions of the experiment may do so under the changed conditions used for detecting viability.

This paper describes a method for investigating the uniformity of inhibition of individual cells in partially inhibited cultures of *E. coli*. Results are given for cells treated with tetracycline, phenol, phenylmercuric acetate and cetyltrimethylammonium bromide.

Experimental

Escherichia coli (NCTC 1013) was used. The conditions of culture, media and methods used to measure absorbance and to prepare cell suspensions have been described previously (Rye & Wiseman, 1966). Freshly prepared solutions of the following antibacterial agents in glucose-free medium were used; phenol B.P., cetyltrimethylammonium bromide (CTAB, cetrimide), phenylmercuric acetate, and tetracycline B.P.

PREPARATION AND TREATMENT OF CELL SUSPENSIONS

Exponentially growing cells were harvested by membrane filtration and suspended in glucose-free medium at 37° to give an absorbance of 0·200.

From the School of Pharmacy, University of Bradford, Bradford 7, England.

Fifteen min after harvesting, equal volumes of the suspensions and of solutions of the antibacterial agents were mixed and maintained at 37°. After a further 15 min, glucose (1 mg/ml) together with sufficient ampicillin B.P. to produce a final concentration of 2 μg/ml were added and the growth of the cells followed by absorbance measurements.

TOTAL CELL COUNTS AND SIZE (VOLUME) DISTRIBUTIONS

These were obtained using a model B Coulter electronic particle counter at various times after the addition of glucose and ampicillin to the treated suspensions. Details of the methods used have been described previously (Rye & Wiseman, 1967a). Size distributions are represented graphically or characterized by the parameters mode, median and mean cell volumes and coefficient of variation. The coefficient of variation is the ratio of the calculated standard deviation of the size distribution to the mean cell volume and is a measure of the spread of cell sizes in a suspension (Koch, 1966).

Results

Preliminary experiments were performed to determine the concentrations of tetracycline, phenol, phenylmercuric acetate and CTAB required to partially inhibit the growth of cultures of *E. coli*. The addition of 2 μg/ml of ampicillin to such treated cultures and to untreated controls was found to prevent cellular division but to have no significant effect upon the rates of increase in absorbance. No evidence of cell lysis was observed in any of the experiments reported.

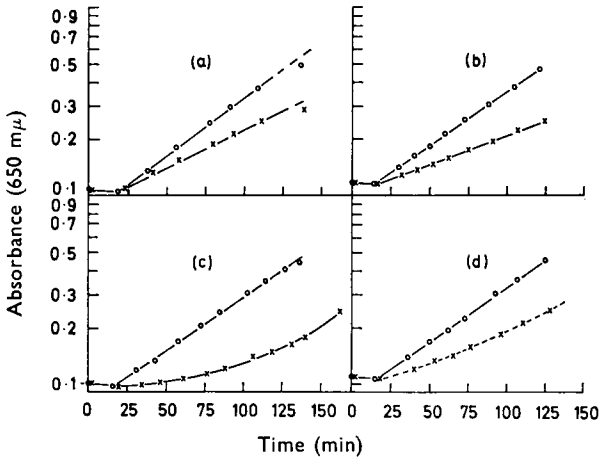

FIG. 1. Changes in the absorbance of suspensions of *E. coli* in glucose-free medium at 37° after the addition of glucose (1 mg/ml) and ampicillin (2 μg/ml) in the absence ○——○ and the presence ×– – –× of (a) 0·25 μg/ml tetracycline, (b) 1·0 mg/ml phenol, (c) 0·03, μg/ml phenylmercuric acetate and (d) 1 μg/ml CTAB. These antibacterial agents were added at time zero and the glucose and ampicillin after 15 min. The broken line in (d) is the theoretical change in absorbance calculated using equation (1) with $\alpha = 0{\cdot}4$ and $k^1 = k$.

Fig. 1 shows the changes in absorbance which occurred after the addition of glucose (1 mg/ml) and ampicillin (2 μg/ml) to resting suspensions of *E. coli* containing (*a*) 0·25 μg/ml tetracycline, (*b*) 1·0 mg/ml phenol, (*c*) 0·03 μg/ml phenylmercuric acetate and (*d*) 1 μg/ml CTAB, and to untreated control suspensions. Growth in the presence of phenol or tetracycline was exponential but occurred at a slower rate than in the control suspensions. With phenylmercuric acetate or CTAB-treated cells, the graph of log absorbance with time was convex to the time axis.

At various times, measurements were made of the distributions of cell sizes in the suspensions described in Fig. 1 and the results are given in Figs 2–4 and in Table 1.

TABLE 1. THE PARAMETERS OF THE SIZE DISTRIBUTIONS OF THE CONTROL AND PHENOL-TREATED CELLS OF FIG. 1(b), BEFORE AND 80 MIN AFTER THE ADDITION OF GLUCOSE AND AMPICILLIN

	Time (min) after the addition of glucose and ampicillin	Cell volume (μ^3)			Coefficient of variation
		Mean	Median	Mode	
Control cells ..	0	0·93	0·85	0·74	0·351
Control cells ..	80	2·31	2·10	1·80	0·391
Phenol-treated cells	80	1·41	1·32	1·15	0·358

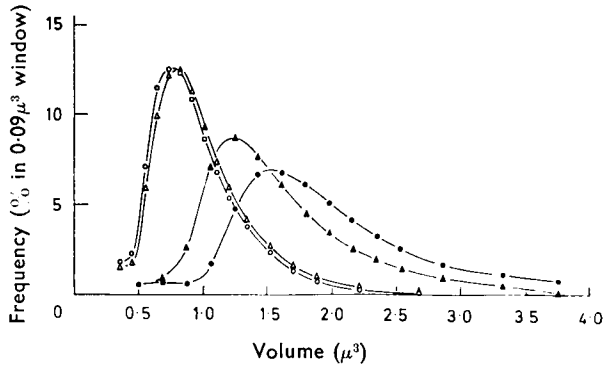

FIG. 2. The size distributions of the control ○——○ and tetracycline-treated cells △——△ of Fig. 1(a) before (open symbols) and 60 min after (full symbols) the addition of glucose and ampicillin.

Fig. 2 compares the cell size distributions of tetracycline-treated cells with those of the control suspension before and 60 min after the addition of glucose and ampicillin to the cells. During this period the mean cell size of the tetracycline-treated cells increased from 1·00 to 1·62 μ^3 and that of the control from 0·97 to 1·99 μ^3. No significant change in the coefficients of variation of the size distributions occurred, the values changing from 0·387 to 0·374 and 0·385 to 0·375 for the tetracycline-treated cells and controls respectively. In both cultures virtually no cells were left in the smallest size ranges after 60 min.

Similar results were obtained with phenol-treated cells and Table 1 compares the parameters of the size distributions obtained after 80 min

growth in the presence of 1·0 mg/ml phenol with those of the control suspension.

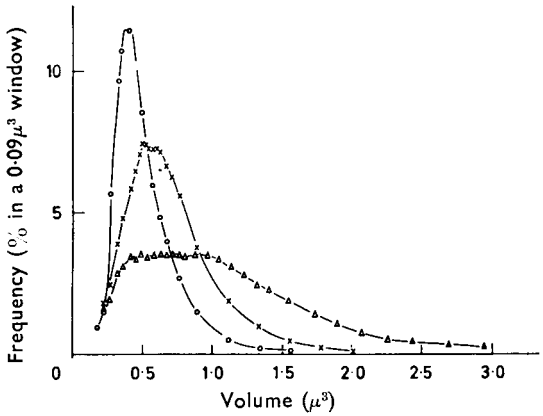

FIG. 3. The size distributions of the phenylmercuric acetate-treated cells of Fig. 1(c), before ○——○, 100 min ×——×, 220 min △——△ after the addition of glucose and ampicillin.

Fig. 3 shows the size distributions of phenylmercuric acetate-treated cells before and 100 and 220 min after the addition of glucose and ampicillin. Both the mean cell volume and the coefficients of variation of the distributions increased during growth, and even after 220 min some cells still remained in the smallest size ranges.

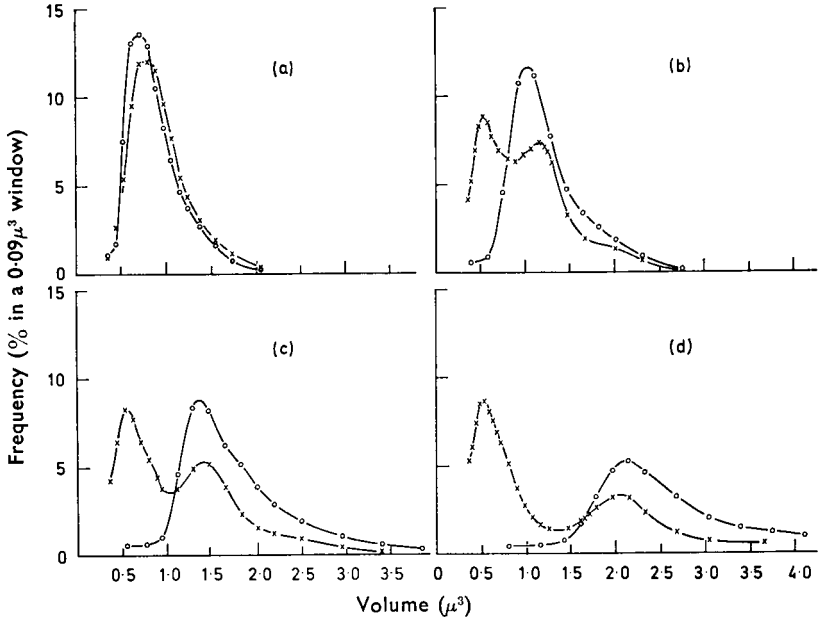

FIG. 4. The size distributions of the control ○——○ and CTAB-treated cells ×——× of Fig. 1(d), (a) before, (b) 34, (c) 60 and (d) 95 min after the addition of glucose and ampicillin.

Fig. 4 shows the size distributions of control and CTAB-treated suspensions (*a*) before, (*b*) 34, (*c*) 60 and (*d*) 95 min after the addition of glucose and ampicillin. The mean cell size of the control suspension increased throughout this period with little increase in the coefficient of variation. In the CTAB-treated suspension, the size distribution widened during growth and resolved itself into a bimodal distribution. From these distributions it was calculated that approximately 60% of the cells had remained at their original size whilst the remainder had increased in size at about the same rate as those in the control suspension.

Discussion

Kinetic studies of the effects of antibacterial agents on micro-organisms at concentrations below those required for the total inhibition of cellular growth can yield information on their mechanisms of action (Garrett, Miller & Brown, 1966). Studies using chloramphenicol and tetracycline indicated that the overall growth rate constant of cultures is reduced by low concentrations of these antibiotics (Ciak & Hahn, 1958). Garrett & Miller (1965) found that the total and viable cell counts coincided during the partially inhibited growth of *E. coli* in the presence of chloramphenicol or tetracycline and they suggested that this resulted from a general inhibition of growth of all of the cells rather than to some of the cells not growing. But with other antibacterial agents, the reduced growth rate of cultures may result from a non-uniform inhibition of the individual cells. This possibility was discussed by Treffers (1956), and Parkinson & Pickett (1964) showed that although the main effect of sub-bacteriostatic concentrations of phenol was to increase the generation time of *E. coli*, some loss in viability occurred in the growing cultures at the higher concentrations above 30°. Studies in this field have previously been complicated by the absence of a method for determining whether or not the individual cells in such partially inhibited cultures are inhibited to the same extent and we believe that this information is essential in order to understand fully the reasons for the overall decrease in growth rate.

The presence of low concentrations of ampicillin prevents the increase in numbers which normally follows the addition of glucose to suspensions of *E. coli* in glucose-free medium but has no effect upon the rate of increase in cell mass (Rye & Wiseman, 1967b). Similar results were obtained during this investigation when ampicillin was added to cultures partially inhibited by tetracycline, phenol, phenylmercuric acetate or CTAB. In these cultures where cellular division is prevented by ampicillin, those cells that are able to grow simply increase in size at rates dependent on their individual growth rates. After some growth has occurred, a comparison of the size distribution of these partially inhibited cells with that of a control suspension containing ampicillin alone, gives information both on the overall extent of growth and on the distribution of the individual cell growth rates in the presence of these antibacterial agents.

The coefficients of variation of the size distributions obtained when using phenol- or tetracycline-treated cells are no greater than in the control

suspensions. This indicates that the decreased overall growth of these cultures results from an extremely uniform inhibition of growth of all the individual cells. In the culture treated with phenylmercuric acetate however, the gradual widening of the size distribution and the continual but slow decrease in the number of cells in the lower size ranges suggests that all the cells are growing but at widely different rates. Phenylmercuric acetate thus appears to inhibit the individual cells in a culture to different extents either by causing a non-uniform decrease in their rates of growth or by imposing a lag period on the cells the duration of which varies from cell to cell. The bimodal distribution of cell sizes developing in the presence of CTAB shows the presence of two kinds of cell. The smaller cells which retain the size characteristic of glucose-starved cells are clearly not growing, whereas the larger cells appear to be growing at the same rate as the untreated control cells. The relative number of the two types of cell remained constant and it was estimated from the distributions that 60% of the cells were not growing. The partially inhibited growth of CTAB-treated cultures unlike that of tetracycline- or phenol-treated cultures thus results from a reduction in the actual number of cells growing and not from a general decrease in the growth rate of the cells.

The results which we have described in this paper suggest that the growth of bacterial cultures may be represented by the equation:

$$D = \alpha D_0 e^{k't} + (1 - \alpha) D_0 \quad .. \quad .. \quad (1)$$

where D_0 represents the total bacterial population in terms of either mass or numbers and D the total population after time t, α is the fraction of the initial population that is actually growing, and k^1 is a growth rate constant for mass or numbers as appropriate. In an uninhibited, exponentially growing culture $\alpha = 1$ and k^1 becomes the first order growth rate constant k. The equation then simplifies to the generally accepted growth equation:

$$D = D_0 e^{kt} \quad .. \quad .. \quad .. \quad .. \quad (2)$$

When growth is partially inhibited by the addition of an antibacterial agent, the overall decrease in growth rate may be due to one or both of two effects: (1) the complete inhibition of growth of some of the population i.e., a decrease in α, and (2) a decrease in the value of the growth rate constant k.

If the values of α or k^1 alter as the bacterial population increases, equation (1) will become inapplicable.

The chief factor governing the type of growth inhibition which occurs may be the extent of uptake of the antibacterial agent being used. Thus if only a small proportion of the antibacterial agent is adsorbed out of solution by the cells then the increase in cell population during growth will cause little or no change in its effective concentration and the inhibiting effects will remain constant with time. It is probable that in these circumstances the agent will be uniformly distributed amongst the cells and each cell will be inhibited to about the same extent. Where however most of the agent is taken out of solution by the cells it may be unevenly distributed amongst the cell population and the individual cells

inhibited to different extents. In addition, any growth which occurs will reduce the effective concentration of agent per unit of cell population and if the agent is not firmly bound then a decrease in the number of cells inhibited or an increase in their growth rate constants may occur.

Under the conditions of our experiments tetracycline and phenol act solely by decreasing the value of the growth rate constant, their effects are relatively uniform on all the cells and are in no way reduced by the increase in cell population during the first 2 hr of growth. These observations suggest that the proportion of these agents taken up is small at the concentrations used. The data presented by Judis (1964) and Bean & Das (1966) for the uptake of phenol confirm this conclusion. CTAB however decreases the proportion of the cell population able to grow and, as the number of inhibited cells did not decrease during the time span of our experiments, its uptake and inhibitory effects appear to be irreversible. The results obtained by McQuillen (1950) and Salton (1951) for the uptake of CTAB by bacteria indicate that at the low concentrations used in our experiments most of the agent would be taken up by the cells. McQuillen (1950) also showed that in dilute solutions of this agent the electrophoretic mobilities of the individual cells were affected to different extents. Phenylmercuric acetate affects all the cells in a culture but to different extents and as α and k^1 both appear to alter during growth its effects must be reversible.

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