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The effects of some tetracyclines on synchronously growing cultures of *Escherichia coli* B/r

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Samples of synchronously growing cultures of *Escherichia coli B/r* removed at predetermined time intervals after synchronization were added to solutions containing bactericidal concentrations of tetracycline. Three active antibacterials, 7-nitro-6-demethyl-6-deoxytetracycline, 9-amino-6-demethyl-6-deoxytetracycline and 6-demethyl-6-deoxytetracycline, and one inactive compound, 7-chloroisotetracycline, were studied. Survival curves for the active agents, determined using samples of culture differing in age, were of constant slope for a given tetracycline. It is concluded that interruption of protein synthesis is the primary mode of action at bactericidal concentrations of tetracyclines, the same process as has been established for the action of the tetracyclines at the lower bacteriostatic concentrations.

Bacterial growth kinetics have been used to assess quantitatively the relative potencies of various antibacterials and also to provide insight into the possible mechanisms for action of these agents (Brown & Garrett, 1964; Jones & Morrison, 1962). Studies on tetracyclines over a wide range of concentrations suggest that at bacteriostatic concentrations these antibiotics function in one way while at bactericidal concentrations they function in another way. This suggestion is based on the observation that bacterial inhibition in the presence of bacteriostatic concentrations follows a different rate law than bacterial inactivation in the presence of bactericidal concentrations (Brown & Garrett, 1964). For low concentrations of drug there is general agreement that the primary mechanism of action is an inhibition of protein synthesis (Weisblum & Davies, 1968). With high concentrations of drug, however, evidence has been presented suggesting that inhibition of cell wall synthesis (Park, 1958), of components of the respiratory chain (Laskin, 1967), or of other biochemical systems (Laskin, 1967; Snell & Cheng, 1959), may contribute to the bactericidal effects of the tetracyclines.

Recently a number of reports dealing with the effects of various antibacterials on synchronously growing bacterial cells have appeared in the literature (Mathison, 1968; Srivastava & Thompson, 1966; Srivastava & Thompson, 1968). Cells in synchronous cultures are essentially all at the same stage in the division cycle making this technique most attractive for investigating the mode of action of the tetracyclines under bactericidal conditions. When penicillin or phenol is added as the antibacterial agent to synchronous cultures of differing age, a periodic increase and decrease in the values for the slopes of the survival curves is noted, indicating changes in the mode of action of each agent at different stages of the growth cycle (Mathison,

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1968; Srivastava & Thompson, 1966). We have looked for differences (not necessarily periodic) in the slopes of survival curves for *Escherichia coli* that could be interpreted as reflecting the inhibition of protein synthesis and one or more additional biochemical processes by tetracyclines.

EXPERIMENTAL

Materials and methods

Organism. *Escherichia coli B/r*, kindly donated by Dr. N. Yamamoto of Fels Research Institute, was used as the test organism.

Chemicals. The tetracyclines used were provided as hydrochlorides by Dr. J. H. Boothe of Lederle Laboratories. All materials used in the preparation of the media and the buffer solutions were Baker Analyzed Reagent Grade Chemicals. Agar was obtained from Bacto-Difco. The water for all solutions was distilled from an all-glass still.

Media. The liquid growth medium was K_2HPO_4 (7 g), KH_2PO_4 (3 g), $(NH_4)_2SO_4$ (3 g), $MgSO_4$ (0·08 g), Na_2SO_4 (8·5 g), Na-citrate (0·5 g), dextrose (10 g), water to 1000 ml. This was sterilized by filtration through a 0·22 μm Millipore filter under positive pressure. Solid medium was prepared by adding sterile liquid agar (sterilized by autoclaving at 15 p.s.i. for 20 min) with aseptic precautions to sterile liquid growth medium.

Diluent. A sterile phosphate buffer solution, K_2HPO_4 (7 g) and KH_2PO_4 (3 g) in 1000 ml of water (pH 7·2), was used to make dilutions for the viable cell counts.

Electrolyte solution. Suspensions of the organisms on which total counts were made using the Coulter Counter were diluted with NaCl (0·9% w/v) solution containing 0·2% (w/v) formaldehyde. This solution was filtered through two Millipore filters one of pore size 0·45 μm superimposed on one of pore size 0·22 μm for sterilization and removal of particles. The formaldehyde was included to prevent growth after sampling.

Total count method. Samples of bacterial suspension (1 ml) were diluted with electrolyte and the cells counted using a Model B Coulter Counter fitted with a 30 μm aperture tube. The operating conditions for the instrument were aperture current 0·707, amplification 1/4, lower threshold 10, upper threshold out.

Viable counting method. A spread plate technique was used. Replicate samples (0·3 ml) of suitably diluted bacterial suspensions were pipetted onto each of five overdried agar plates. The dilutions were made to obtain a maximum plate count of 500 colonies. The plates were incubated at 37° for 24 h before counting. The counts were made under direct illumination with magnification and all plates were subsequently re-examined after a further 72 h incubation for the appearance of new colonies.

Synchronization technique. Synchronized cultures were obtained using a modification of the procedure of Anderson & Pettijohn (1960). A sample of a 17 h culture was added to fresh medium at 37·5° and allowed to grow until the total count was 4×10^7 organisms/ml. This culture was then filtered under positive pressure through two 3-μm Millipore filters, one above the other, and the filtrate collected in a sterile

flask at 37·5°. The most consistent results were obtained when the filters were pre-moistened with sterile water.

Determination of bactericidal concentration. Graded concentrations (0–60 μg/ml) of each tetracycline were added to synchronous cultures and the survivors estimated by plate counts. The concentration chosen was that which reduced the number of viable organisms by a factor of 100 within 60 min (20 μg/ml).

Determination of bactericidal kinetics. Samples of a synchronized culture were removed at pre-determined times after synchronization and to these were added liquid medium containing tetracycline (at 37·5°) to give a final concentration of 20 μg/ml of drug. At timed intervals, these cultures, which were maintained at 37·5°, were sampled (1 ml) and after appropriate dilution pipetted onto agar. The number of viable organisms was estimated according to the procedure given earlier.

RESULTS

Synchronized cultures of *E. coli* at 37·5° had a doubling time of 57 min when grown in the liquid media. The synchrony could be maintained through two divisions. A representative example of the synchronization achieved is shown in Fig. 1a. Tetra-

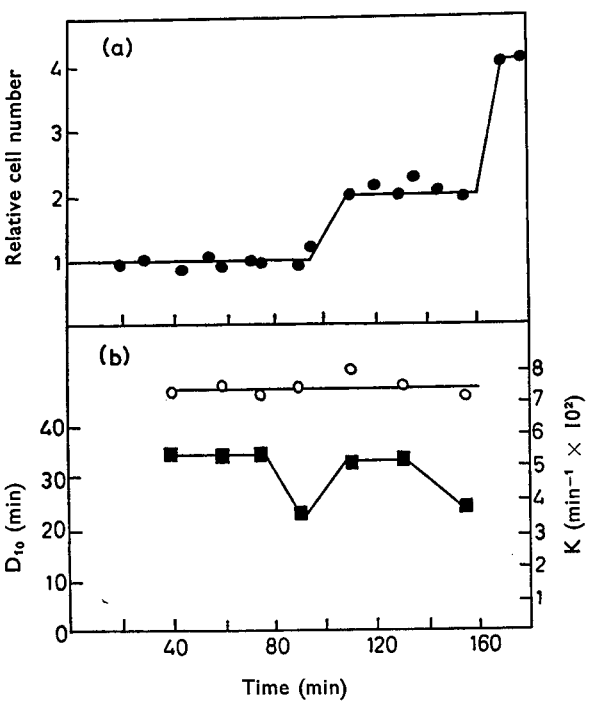

FIG. 1. (a) Cell number relative to initial innoculum at different times after synchronization; (b) Values of D_{10} (■) and apparent inactivation constants k (○) for *E. coli* B/r in the presence of 20 μg/ml of the 7-nitro-tetracycline analogue at different times after synchronization.

cyclines were not added to cultures until 30 min after synchronization since an induced lag of 30 min was consistently observed. Fig. 2 shows typical survival curves for samples of cultures exposed to the 7-nitro-substituted compound at various

times after synchronization. Survival curves for an individual tetracycline exhibited a constant lag period between the time the tetracycline was introduced into the culture sample and the time the bactericidal effects were first noted. This lag period was much shorter for those samples of culture which were taken within 5 min of division; the lag decreased by 50, 40 and 30% for the 7-nitro, 9-amino, and unsubstituted tetracyclines, respectively.

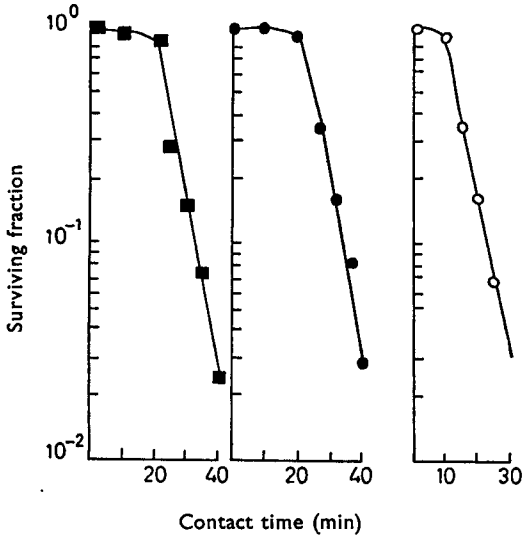

FIG. 2. Typical survival curves for synchronous *E. coli* B/r in the presence of the 7-nitro-tetracycline analogue: (■) 40 min, (●) 75 min and (○) 90 min after synchronization.

The linear portions of the survival curves were fitted by least squares regression techniques to the equation

$$\log (S/S_0) = -k\,t + \log n \qquad (1)$$

where S/S_0 is the fraction of viable cells remaining after a given contact time t, k is the apparent first-order inactivation rate constant and log n is the intercept of the extrapolated linear portion of the curve with the y-axis. A measure of the sensitivity of a given sample of culture towards the antibiotic may be expressed in terms of D_{10}, i.e. the time at which 10% of the total bacterial population still survives. Values for k, n and D_{10} appropriate to each of the tetracyclines investigated are given in Table 1.

For an individual tetracycline, the values for k did not change, showing the death rate was similar throughout the division cycle. Fig. 1b shows the relation between the age of culture after synchronization and the values for k obtained for the 7-nitro-substituted tetracycline. The values of k are in the ratio 1 : 1·57 : 2·06 for the unsubstituted, the 9-amino- and the 7-nitro-substituted tetracyclines, respectively. If the inhibition rate constants obtained by Miller, Kahlil & Martin (1970) are extrapolated to the concentration used in this study, the corresponding ratios are 1 : 1·5 : 7·9. Within the limits that the extrapolation can be considered valid, it seems that a 7-nitro-substituted tetracycline is more potent as a bacteriostatic agent than as a bactericidal agent.

Table 1. *Survival curve parameters for different tetracyclines on synchronously growing cultures of* Escherichia coli B/r *and bactericidal potencies and* π *values*

Age of culture	Tetracycline analogue[a]								
	7-Nitro-			9-Amino-			Unsubstituted		
min	D_{10}[d]	n[b]	k[c]	D_{10}[d]	n[b]	k[c]	D_{10}[d]	n[b]	k[c]
40	—	—	—	41	1·43	5·36	63	1·31	3·67
45	34	1·33	7·13	—	—	—	—	—	—
55	—	—	—	46	1·43	5·24	62	1·36	3·82
60	34	1·53	7·72	—	—	—	—	—	—
75	35	1·37	7·19	47	1·71	5·64	63	1·37	3·76
90	23	0·69	7·48	30	1·09	5·66	55	0·63	3·02
110	32	1·65	8·14	46	1·71	5·92	65	1·68	4·19
130	34	1·51	7·67	—	—	—	—	—	—
135	—	—	—	47	1·52	5·37	66	1·13	3·38
155	25	0·59	6·87	38	0·97	5·18	37	0·45	3·42
Mean value of Inactivation constant			7·45 (±0·41)			5·48 (±0·27)			3·61 (±0·37)
π			0·50			—1·10			0·00

a. 7-Chloroisotetracycline had no detectable effect on the cultures.

b. These values are to be considered as tentative since the experimental design was not ideal for the determination of extrapolation values.

c. In units of min^{-1} ($\times 10^2$).

d. In units of $min.^{-1}$

Based on D_{10} values, the sensitivity of the culture samples towards a tetracycline was unaffected by the age of the culture until 5 min before division. At this point the sensitivity increased by 23, 33 and 33% for the unsubstituted, the 9-amino- and the 7-nitro-substituted tetracyclines, respectively. The increase in sensitivity, *i.e.* the decrease in D_{10}, near division is shown in Fig. 1b for the 7-nitro-substituted tetracycline.

At times other than within 5 min of division, the mean values for n (eqn 1) are 1·37, 1·56 and 1·48 for the unsubstituted, the 9-amino- and the 7-nitro-substituted analogues, respectively. While the experimental design was not ideal for the estimation of these values (Porter, 1963) the similarity in the extrapolation values suggests that the tetracyclines tested have a common mode of action at the concentration used. Lower extrapolation values are observed for cultures that are within 5 min of division, and the similarity in the values for n at this stage is most evident for the unsubstituted and the 7-nitro-substituted compounds. The greater value for n noted at this stage for the 9-amino-derivative indicates that in this instance the culture was further from division than were the cultures in the other two instances.

DISCUSSION

From a kinetic standpoint, survival curves that differ in slope for samples of culture taken at various stages in the division cycle could be taken as evidence for at least two modes of antibacterial action. With the tetracyclines the slopes of the survival curves for a given tetracycline are essentially the same at all stages in the growth of the synchronous culture. Thus, it seems likely that the bactericidal effects of the tetracyclines are produced by the inactivation of a single biochemical process

that is continuous throughout the cell cycle. As tetracyclines in bacteriostatic concentrations are known to inhibit protein synthesis (Laskin, 1967; Snell & Cheng, 1959; Weisblum & Davies, 1968), and protein synthesis occurs continually throughout the cell cycle (Maaløe & Kjeldgaard, 1966), it appears that the bactericidal effects of the tetracyclines arise primarily because of an inactivation of protein synthesis. It is suggested that an inhibition (short of total inactivation) of protein synthesis by tetracyclines leads to bacteriostasis while the inactivation of protein synthesis by tetracyclines affords a bactericidal effect.

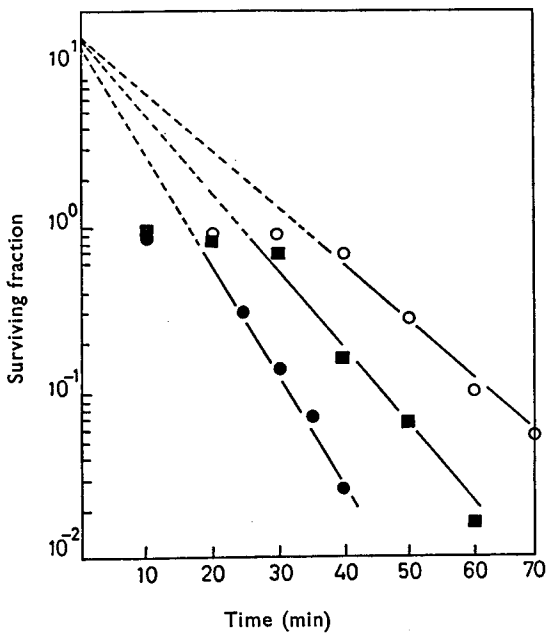

FIG. 3. Superposition of survival curves for synchronous cultures of *E. coli* B/r in the presence of 20 μg/ml, 7-nitro- (●), the 9-amino- (■) and the unsubstituted (○) tetracycline analogues, at times other than within 5 min of division.

The intercept of the linear portion of the survival curve with the line representing the initial population number (Fig. 3) may be regarded as the lag period between the time a synchronous culture is first placed in contact with a solution containing tetracycline and the ideal time when the total bacterial population first experiences the bactericidal effects of the tetracyclines. This lag time may be interpreted as the period during which a pool of available protein is utilized. It is longer for the less potent tetracyclines since these compounds are inefficient inhibitors of protein synthesis. Near division, the lag time is shortened and this reflects an increase in the rate at which protein is used. In support of this interpretation are four observations: (1) the accepted primary mode of tetracycline action is the inhibition of aminoacyl-tRNA addition to the 30s ribosomal subunit (Weisblum & Davies, 1968); (2) the bacteriostatic and bactericidal potencies of the tetracyclines are linearly related (Cammarata, Yau & others, 1969); (3) RNA synthesis continues at an unreduced rate after protein synthesis has been interrupted and diminishes in rate as the pool

of available protein becomes exhausted (Maaløe & Kjeldgaard, 1966); (4) the rate of RNA synthesis increases at division (Maaløe & Kjeldgaard, 1966).

It could be argued that the differing lag times observed for each tetracycline may be a consequence of their relative ability to penetrate the bacterial cell wall. The diminished lag time near division would then be a consequence of the increased porosity of the cell wall towards the tetracyclines at this stage. However, the slopes of the survival curves k are inversely proportional to the lag times, and there was no correlation between these inactivation constants and the π values for the substituents. [π is a measure of the lipophilicity imparted by a substituent and is defined by $\pi = \log P_X - \log P_H$ where P is the partition coefficient for a substituted member X and the parent H of a congeneric series (Hansch, 1968.)] A similar lack of correlation between the bacteriostatic potencies for 11 tetracyclines and π has been noted by Cammarata & Yau (unpublished). On this basis it appears that passive transport of the tetracyclines through the bacterial cell wall is not a primary factor determining the lag time.

Rate determining active transport through the bacterial cell wall could also account for the differing lag times, but, near division, either an increase in the rate of active transport or a change in the rate determining step must take place and the net effect would be to decrease the lag time. Then the overall mechanism of action would be changed near division and should be reflected by a change in the slope of the survival curve at this stage. Since no such change is observed, it appears likely that the inhibition of protein synthesis is the rate determining step which affects the lag time.

The lag periods reported by Mathison (1968) for chloramphenicol differ from those reported here in that his lag periods varied with stage while with the tetracyclines the lag is essentially invariant with stage up to division. It is possible that this difference may be a consequence of the different sites of action of these two antibiotics at the ribosomal level (Weisblum & Davies, 1968).

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