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Bacteriostatic actions of some tetracyclines

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6-Demethyl-6-deoxytetracycline, 6-methyleneoxytetracycline, 7-chlortetracycline and 7-chlor-6-demethyltetracycline inhibit *Aerobacter aerogenes* in glucose-mineral salt media, by two modes of action, I and II, shown by tetracycline and oxytetracycline (Jones & Morrison, 1962; 1963): Mode II by 6-demethyl-6-deoxytetracycline is not detected by inhibition of growth of the organism but by inhibition of the anaerobic consumption of pyruvate which is also a consequence of Mode II. The chlorine-containing tetracyclines also inhibit the growth of the organism by a third mode of action (III) which is probably an interference with the provision of D-glutamate during aerobic growth. The inhibitions produced by a tetracycline are linearly related to the amount of the antibiotic in the molecular form present in the cultures. All three modes of action so far described are probably interferences with hydrogen transfer reactions. The bacteriostatic activities of six tetracyclines are compared.

IT has been shown by Jones & Morrison (1962; 1963), using a quantitative assessment of inhibition of the rate of growth of bacteria (Harris & Morrison, 1961), that tetracycline and oxytetracycline interfere with the metabolism of *Aerobacter aerogenes* in two ways and that only one of these modes of action limits the rate of growth of the organism under any one set of conditions. Inhibition of the rate of growth by both modes is linearly dependent on the concentration in the culture of the molecular form of the antibiotic and not on the concentrations of its ionic forms or of its complex with magnesium ions. Mode I was identified as a derangement of protein synthesis; Mode II as an interference with the transfer of hydrogen or the production of a hydrogen acceptor required in unaerated cultures. The inhibition of the rate of consumption of pyruvate by non-growing cells under the same conditions was also accounted for as a consequence of Mode II. Equimolecular concentrations of the molecular forms of the two tetracyclines are equally effective by Mode I but not by Mode II.

The simpler tetracycline, 6-demethyl-6-deoxytetracycline, and also 6-methyleneoxytetracycline, 7-chlortetracycline and 6-demethyl-7-chlortetracycline have now been used to examine further the structure-action relationships in this group of antibiotics. The primary sites of inhibition corresponding to each mode have been identified more closely and a new mode discovered.

Experimental and results

ORGANISM AND MEDIA

The organism, media and temperature of incubation, 37° ± 0·1°, are the same as those used previously (Jones & Morrison, 1962). Since 7-chlortetracycline is less stable in aqueous solution than the tetracylines previously examined, instead of being introduced before inoculation the antibiotics were added to the growing cultures just before growth was adequate enough to be measured optically. Inocula were taken from the third of three cultures grown in rapid succession in media containing only sufficient glucose to permit two-thirds of the amount of growth obtained in

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a normal culture. The slopes of the plot of Index Ratio (the mean generation time of the inhibited culture divided by the mean generation time of a non-inhibited culture: Harris & Morrison, 1961), against concentration of inhibitor in culture, in the "basic" medium at pH 7·0, have a maximum deviation of $\pm$ 5% from that of the ideal plot calculated from the results of fourteen experiments. As the concentrations of antibiotic needed were small and had to be obtained by serial dilution, in each subsequent experiment involving changes of conditions and a freshly made up solution of the antibiotic, one culture in "basic" medium (Jones & Morrison, 1962) at pH 7·0 was used as an additional control so that any deviation from the intended concentration could be determined from the ideal plot.

ANTIBIOTICS

The nomenclature used is essentially that of Jones & Morrison (1962); $TH_3^+Cl^-$ denotes the hydrochloride of a tetracycline, TH_2 the molecular form, TH^- and T^{2-} the other ionic forms and $MgTH^+$ the complex with magnesium ion, the particular tetracycline being declared in the text when necessary. The dissociation constants and the association constants for the complexes with magnesium ions of tetracycline and oxytetracycline have been listed previously (Jones & Morrison, 1962); those for the other tetracyclines, calculated from potentiometric data by standard methods, are given in Table 1. The potentiometric titration curves indicate that complex formation is between the singly charged TH^- and the Mg^{2+} ions.

EFFECT OF CONCENTRATION OF ANTIBIOTIC UPON GROWTH

In "basic" medium initially at pH 7·0, increasing concentrations of all the antibiotics caused a progressive increase of mean generation time of both aerated and unaerated cultures. The results of plotting the calculated Index Ratios against the concentrations of antibiotic are shown in Fig. 1. In the instances of the 7-chlor-substituted tetracyclines the mean generation times decreased sharply after about 300 min; measurements of rate of logarithmic growth before this change were reproducible.

Growth in aerated cultures. The results for aerated cultures in "basic" medium (in which the four new tetracyclines showed only a single mode of action) indicated how much less potent was the 6-demethyl-6-deoxytetracycline (Fig. 1). When the "basic" medium was enriched with a mixture of amino-acids however, the chlorine-containing tetracyclines showed diminished potency; an effect also obtained in medium enriched with D-glutamate only (L-glutamate had no effect). The inhibition of growth by the chlorine-containing tetracyclines in "basic" medium was not due to either Mode I or Mode II and hence a new mode, Mode III, concerned with the availability of D-glutamate, has to be postulated.

The inhibiting powers of 6-demethyl-6-deoxytetracycline and of 6-methyleneoxytetracycline in aerated "basic" medium, and of the chlorine-containing tetracyclines in aerated "basic" medium enriched with casein hydrolysate, were unaffected by enrichment of the media with bacteriological peptone (Difco) or with yeast-extract (Difco) and thus corresponded to the inhibition produced by the Mode I of tetracycline and oxytetracycline (Jones & Morrison, 1962).

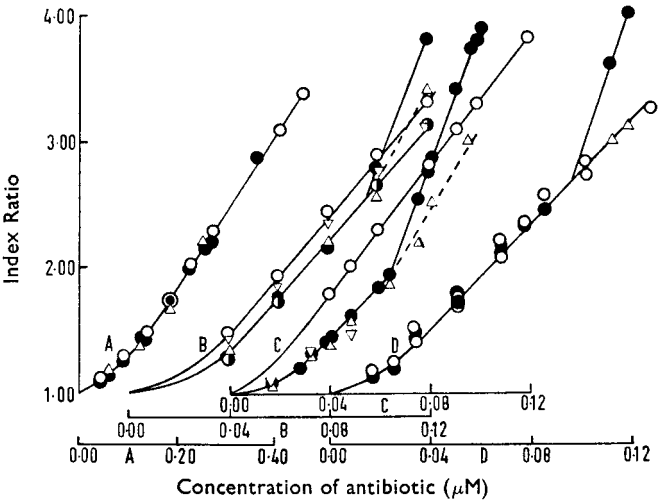


FIG. 1. Inhibition of cultures of *A. aerogenes* by some tetracyclines. Index Ratio (the mean generation time of the inhibited culture) is divided by the mean generation time of the uninhibited culture. A, 6-demethyl-6-deoxytetracycline; B, 7-chlortetracycline; C, 6-demethyl-7-chlortetracycline; D, 6-methyleneoxytetracycline. Unaerated cultures, addition: ●, none; △, 0·05% w/v yeast extract. Aerated cultures, addition: ○, none; ◑, 0·2% w/v casein hydrolysate; ▽, 1·1% w/v glutamate.

Growth in unaerated cultures. At all concentrations of 6-demethyl-6-deoxytetracycline and below concentrations (μM) of 0·093 for 7-chlortetracycline, 0·063 for 6-demethyl-7-chlorotetracycline and 0·096 for 6-methyleneoxytetracycline, enrichment of the medium with yeast-extract had no effect on inhibition. This indication that inhibition was by Mode I was confirmed by the fact that the ratio of the amount of nitrogen utilised to amount of cells produced was greater initially in the inhibited cultures; this is as expected if the utilisation of amino-acids was being inhibited (Jones & Morrison, 1962).

The chlorine- and methylene- substituted tetracyclines at higher concentrations than those in the preceding paragraph, inhibited the rate of growth of cultures in unaerated medium more severely than by Mode I, but inhibition was by Mode I in medium enriched with yeast-extract. Thus inhibition by these concentrations in unaerated cultures corresponded to that produced by the Mode II of tetracycline and oxytetracycline (Jones & Morrison, 1962).

pH OF THE MEDIUM AND INHIBITION

The inhibition of growing cultures caused by a particular *total* concentration—the sum of the concentrations of all the forms of the tetracycline present—of any of the four tetracyclines, irrespective of which mode was operative, was increased by lowering the pH of the medium and decreased by increasing the pH. This can be effected by varying the concentration of sodium ions in the medium. An example illustrating this general finding (Benbough, to be published) is given in Table 2. The severity of

TABLE 1. IONISATION CONSTANTS (K_1, K_2, K_3) OF SOME TETRACYCLINES AND STABILITY CONSTANTS (K_S) OF THEIR COMPLEXES WITH MAGNESIUM

	6-Demethyl-6-deoxytetracycline	6-Methylene-tetracycline	7-Chlor-tetracycline	6-Demethyl-7-chlortetracycline
pK_1	3·45	3·11	3·57	3·36
pK_2	7·87	7·61	7·57	7·37
pK_3	9·67	9·64	9·53	9·62
$\log_{10}K_S$	4·95	4·19	4·41	4·17

TABLE 2. INHIBITION BY 6-DEMETHYL-6-DEOXYTETRACYCLINE IN UNAERATED MEDIA INITIALLY AT pH 6.20, 7·00 AND 7·80

Concentration of antibiotic (μM)	0·044	0·062	0·089	0·124	0·133	0·178	0·187	0·220	0·249	0·306
Index ratio ± 0·08										
at pH 6·20	1·23		1·46		1·94	2·16		2·50		
at pH 7·00	1·11		1·27		1·51	1·75		2·03		
at pH 7·80		1·05		1·23			1·42		1·68	2·32

inhibition associated with a particular concentration of the *molecular* form of a tetracycline, calculated from the data in Table 1, was independent of the initial pH of the medium except with 7-chlortetracycline in aerated cultures at pH 7·80 and 7-chlor-6-demethyltetracycline in both aerated and unaerated cultures at pH 7·80, when the inhibitions were apparently more severe than in the corresponding cultures at pH 7·00. However, the pH of a culture decreases as growth takes place because growth is accompanied by the production of acid (Jones & Morrison, 1963); and the proportion of the molecular form of a tetracycline present in the total concentration should be calculated for the pH prevailing when the rates of growth were measured and not for that prevailing when the culture was inoculated. The errors due to using the initial pH are not significant for the tetracyclines so far examined except the chlorine-substituted ones when the pH is relatively high. If it is assumed that the pH of cultures initially at pH 7·80 had decreased to 7·55 or 7·60 when the measurements of growth rate were made, the results for cultures initially at pH 7·80 and pH 7·00 inhibited by one of the chlorine-substituted tetracyclines are concurrent.

Inhibitions by the four tetracyclines as a function of concentration of molecular form are compared with each other and with tetracycline and oxytetracycline in Figs 2 and 3. Inhibition by Mode I due to tetracycline, oxytetracycline and 6-demethyl-6-deoxytetracycline are not distinguishable; neither are inhibitions due to Mode I for 7-chlor-6-demethyltetracycline and 6-methyleneoxytetracycline, with which inhibition by Mode I due to 7-chlortetracycline is almost concurrent; but inhibitions by Mode II differ in threshold concentrations or rates of increase with concentration, or both, as do inhibitions by Mode III.

MAGNESIUM ION CONCENTRATION AND INHIBITION

As with tetracycline and oxytetracycline (Jones & Morrison, 1962), increasing the concentration of magnesium in the medium decreased the severity of the inhibition caused by a particular *total* concentration of one of the tetracyclines irrespective of which mode was operating, provided the

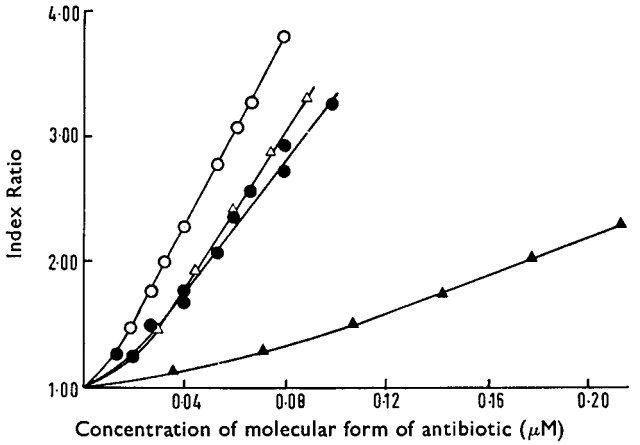


FIG. 2. Comparison of inhibitions of *A. aerogenes* by some tetracyclines in aerated cultures. Cultures inhibited by: 6-demethyl-7-chlortetracycline, ○; 7-chlortetracycline, △; 6-methyleneoxytetracycline, ●; 6-demethyl-6-deoxytetracycline, ▲. The corresponding plots for tetracycline and oxytetracycline (data from Jones & Morrison, 1962) are concurrent with that for 6-demethyl-6-deoxytetracycline (mode I).

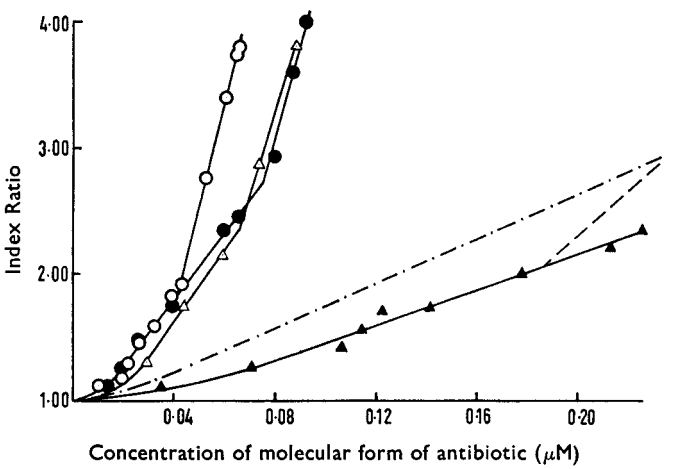


FIG. 3. Comparison of inhibitions of *A. aerogenes* by some tetracyclines in unaerated cultures. Cultures inhibited by: 6-demethyl-7-chlortetracycline, ○; 7-chlortetracycline, △; 6-methyleneoxytetracycline, ●; 6-demethyl-6-deoxytetracycline, ▲. The corresponding plots (data from Jones & Morrison, 1962) for: tetracycline (mode I), concurrent with that for 6-demethyl-6-deoxytetracycline; tetracycline (mode II), — —; oxytetracycline (mode II), — · —.

concentration of magnesium was sufficiently large. An example of this general finding (Benbough, to be published) is given in Table 3. When the measurements were plotted against the calculated concentrations of the molecular species, they were close to the corresponding plots in Figs 2 and 3, which have been drawn from the results obtained in medium initially at pH 7·0 and containing 82μM magnesium. The existence of an apparent

TABLE 3. CONCENTRATION OF MAGNESIUM AND INHIBITION OF UNAERATED CULTURES INITIALLY AT pH 7·0 BY 6-DEMETHYL-7-CHLORTETRACYCLINE

Magnesium concentration (μM)	82	164	820	1650	3300	6600
Index ratio ± 0·08 produced by (μM) of antibiotic:						
0·025	1·22	1·22	1·22	1·15	1·09	1·04
0·040	1·47	1·49	1·47	1·36	1·25	1·12
0·050	1·55	1·58	1·68	1·50	1·37	1·16
0·075	2·68	2·68	2·66	2·16	1·84	1·39
0·100	3·49	3·57	3·42	3·00	2·50	1·67

threshold concentration of magnesium, below which there was no measureable effect on inhibition, was to be expected since low concentrations of magnesium have insignificant effects on the concentrations of the molecular species in the medium; the size of the threshold concentration depends on the values of K_2 and K_S which vary from one tetracycline to another (Table 1). Because of this threshold concentration, with the present limits of accuracy of measuring inhibition, it is impossible to determine whether or not a concentration of magnesium less than 10 μM is necessary for the tetracyclines to be inhibitors.

USE OF ADAPTED STRAINS OF THE ORGANISM

Present work, with that of Jones & Morrison (1962), indicates that the tetracyclines studied may inhibit the rate of growth of the organism because of at least three interferences with its metabolism: Modes I, II and III. This being so, strains of the test organism that are resistant to one tetracycline in that they are unaffected by one or more of its modes of inhibition should show the appropriate cross-resistance to other tetracyclines.

Adapted strains in unaerated medium—Modes I and II. The strain was grown twice in six separate volumes of "basic" medium at pH 7·0, each of which contained a sufficient concentration of one of the tetracyclines to cause inhibition of the rate of growth by Mode II for oxytetracycline and by Mode I for the others. The inhibitions of each growth are given in Table 4. The strain adapted more readily to the 7-chlor- and 6-methylene-

TABLE 4. EFFECT ON INHIBITION OF ONE GROWTH IN THE PRESENCE OF A TETRACYCLINE

Antibiotic	Concentration mμM	Mode	Index ratio ± 0·08	
			1st growth	2nd growth
Tetracycline	83·3	I	1·34	1·34
Oxytetracycline	80·7	II	1·60	1·50
6-Demethyl-6-deoxytetracycline	88·9	I	1·32	1·32
7-Chlortetracycline	77·8	I	2·12	1·58
6-Demethyl-7-chlortetracycline	45·0	I	1·60	1·32
6-Methyleneoxytetracycline	51·9	I	1·71	1·44

substituted tetracyclines than to the others. The two phases of growth in the presence of the 7-chlor- substituted tetracyclines, referred to earlier, is more likely to be due to the readiness of the organism to adapt to these antibiotics than to a loss of antibiotic by degradation in aqueous solution; degradation in aqueous solution is fairly rapid for 7-chlortetracycline but not for 7-chlor-6-demethyltetracycline (Goldberg, 1959; Jones, 1961).

In separate experiments, the organism was grown 10 times in rapid succession in medium containing sufficient of one of the antibiotics to produce an Index Ratio of 3·0 for the first growth (inhibition by Mode II except for 6-demethyl-6-deoxytetracycline which shows only Mode I in growth experiments). This procedure yielded a strain resistant to the tetracycline used at the concentrations that were normally required to produce the inhibitions in unaerated medium shown in Fig. 1. The strains resistant to tetracycline and to 7-chlortetracycline respectively were insensitive also to the presence of the other antibiotics over the ranges of concentration shown in Fig. 1; the cross-resistance to inhibitions by Mode I, or by Mode II, was complete.

Adapted strains in aerated medium—Modes I and III. A strain adapted to become resistant to the presence of sufficient 7-chlortetracycline to produce an Index Ratio of 3·0 in aerated medium by the procedure described in the previous paragraph, also was insensitive to the presence of other tetracyclines in aerated medium in the concentrations shown in Fig. 1. Thus the cross-resistance to inhibitions by Mode I and by Mode III, was complete.

PRIMARY SITES OF INHIBITION BY TETRACYCLINES

Use of redox systems. A strain of *Klebsiella cloacae* will grow readily in aerated basic medium in the presence of the redox dye brilliant cresyl blue, provided the culture is shielded from strong light. Under these conditions phenol, an inhibitor of the organism's normal hydrogen transfer systems, now becomes ineffective (Harris, 1956; Harris & Morrison, 1961). *Aerobacter aerogenes* also grows readily after 3–4 sub-cultures in basic medium containing 0·002% w/v of this dye, aerated cultures having a mean generation time of 30 min and unaerated ones 42 min. In the presence of the dye the organisms were unaffected by any of the six tetracyclines at concentrations which normally produce Index Ratios in excess of 3·0 under the same conditions. There are three possible explanations: (1) the dye caused a rapid alteration of the tetracyclines to inactive forms; (2) the dye itself was used as a by-pass to the parts of the system which are affected by tetracyclines; (3) growth in the presence of the dye resulted in the development of alternative metabolic routes which were not susceptible to the tetracyclines. However, measurements of the concentration of 7-chlortetracycline after incubation in "basic" medium with and without the presence of the dye by the method of Grove & Randall (1955), showed no loss of the antibiotic due to the presence of the dye, and, after growth in medium containing dye, the organisms grew readily in "basic" medium in the absence of dye and were still completely unaffected by normally bacteriostatic concentrations of the tetracyclines. Growth in the presence of the dye must have resulted in the production of a strain which was resistant because of an alteration of metabolism. Similar results were obtained with methylene blue. At concentrations of 7-chlortetracycline greater than 0·2 μM, inhibition reappears even in the presence of brilliant cresyl blue, the Index Ratios being 1·37 and 1·97 respectively at 0·353 and

0·588 μM. This would appear to be a Mode IV, but it has not been investigated further.

Since the redox potentials, E'_0 (V), of brilliant cresyl blue and methylene blue are 0·045 and 0·011 respectively, neither could accept hydrogen from, for example, reduced cytochrome *a* (E'_0, 0·262) but could well do so from reduced cytochrome *b* (E'_0, −0·04) or hydrogen-transferring systems with even more negative E'_0 values.

The effects on inhibitions by tetracyclines of the presence of other redox systems with E'_0 values more positive than that of brilliant cresyl blue, were determined. In aerated cultures, inhibition by 7-chlor-6-demethyltetracycline was unaffected by ascorbic acid (E'_0, 0·204) but was decreased by *o*-cresol-indo-2,6-dichlorophenol (E'_0, 0·188). The amount of the decrease depended on the concentration of the indophenol; 0·0568 μM antibiotic normally produces an Index Ratio of 2·20 by Mode III and in the presence of D-glutamate a value of 1·80 by Mode I, but 20 mg/litre of the redox indicator decreased the value to 1·78 and 60 (or more) mg/litre to 1·59. This indicated that Mode III was an interference with a hydrogen transfer system of E'_0 between 0·188 and 0·204 V, while Mode I was an interference with one of E'_0 approximately 0·188 V.

The effect of *o*-cresol-indo-2,6-dichlorophenol on inhibition of unaerated cultures by 0·25 μM tetracycline was determined. The inhibition was decreased from an Index Ratio of 2·84, expected for inhibition by Mode II, to 1·64, which also was less than would have been expected for Mode I. Thus Mode II was eliminated also, and Mode I was decreased, as found with 6-demethyl-7-chlortetracycline.

Consumption of oxygen. The effects of tetracycline and 7-chlortetracycline on the rates of uptake of oxygen at *p*H 7·0 by suspensions of non-growing cells oxidising glucose (in "basic" medium without the nitrogen source) were measured. Though tetracycline at higher concentrations decreased the rate to, for example, 65% of the normal at 4 μM, within the bacteriostatic range of concentration (0 to 0·4 μM) it stimulated the rate to 109% at 0·05 μM and 103% at 0·4 μM. 7-Chlortetracycline within its bacteriostatic range of concentration (0 to 0·12 μM) also increased this rate and even at 0·19 μM the rate was 104% of normal. Thus although the previous work indicated that Modes I and III were in the hydrogen transfer mechanism of the test organism they could not be direct inhibitions of steps of the mechanism normally used to transfer hydrogen to its ultimate acceptor, oxygen; they must be inhibitions of steps in pathways linking the main hydrogen transfer system to the growth mechanisms, which would not be operating fully when the measurements were made. The stimulations observed indicated that the use of such pathways was inhibited in the bacteriostatic ranges of concentration. If this were so, the efficiency with which glucose is utilised for the synthesis of new cellular material should be impaired by bacteriostatic concentrations.

When the concentration of glucose is limiting, there is a linear or almost linear relationship between the amount of growth and concentration of glucose (Dagley, Dawes & Morrison, 1951). If the efficiency of utilisation of glucose is impaired, the slope of the plot of amount of growth against

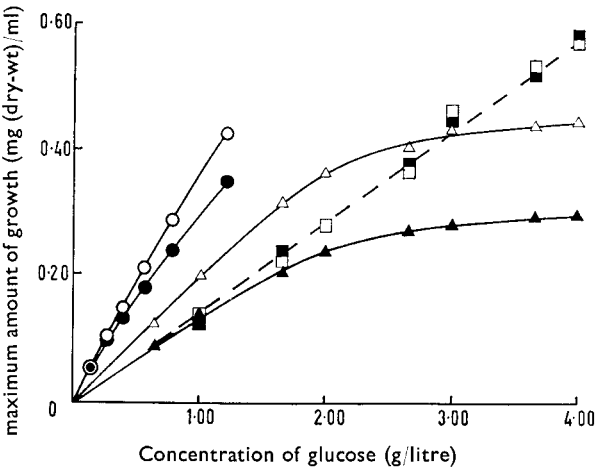


FIG. 4. Effect of 0·1781 μM tetracycline on the efficiency of utilisation of glucose for growth of *A. aerogenes*. Aerated basic media: uninhibited, ○; inhibited, ●. Unaerated basic media: uninhibited, △; inhibited, ▲. Aerated media containing 0·002% w/v brilliant cresyl blue: no tetracycline, □; tetracycline present, ■.

glucose utilised should be decreased. Fig. 4 shows that this is so for tetracycline inhibiting by Modes I and II, and that tetracycline has no effect on the efficiency of utilisation of glucose by a strain which is resistant to the antibiotic.

Consumption of inorganic phosphate. Side pathways linking the main pathway for transfer of hydrogen to oxygen, to the growth mechanism are likely to be associated with phosphorylation. Cells were suspended in aerated isotonic maleic buffer containing: (g/litre); glucose, 10, $MgSO_4$. $7H_2O$, 0·07: (μg/litre); NaH_2PO_4, 155; and various concentrations of tetracycline. The concentration of inorganic phosphate was determined at intervals by the method of Fiske & Subbarow (1927). The eventual amount of inorganic phosphate consumed was lessened, but the initial rate of consumption was stimulated—for example it was increased 13, 44, 94 and 119% by 0·208, 0·417, 2·080 and 4·170 μM tetracycline respectively. Clearly there was no direct inhibition of phosphorylation, and as the rate of uptake of oxygen was decreased by the higher concentrations of tetracycline, the phosphorylation must be associated with a side pathway rather than with the main pathway.

RELATION BETWEEN MODE II AND UTILISATION OF PYRUVATE

Tetracycline and oxytetracycline inhibit the utilisation of pyruvate by non-growing suspensions of cells under the same conditions as they inhibit the rate of growth by Mode II; though the utilisation of pyruvate cannot be the reaction primarily affected by Mode II, it also could be a consequence of Mode II (Jones & Morrison, 1963). This work has been extended to include the four other tetracyclines at pH 7·0. All inhibited the utilisation of pyruvate under unaerated conditions but not if aeration was used; 6-demethyl-6-deoxytetracycline, which does not inhibit rate of growth by

Mode II, inhibited the pyruvate utilisation much less severely. In each instance after a threshold concentration of the antibiotic, the plot of the reciprocal of the rate of consumption of pyruvate against concentration of antibiotic is linear, as it is for the previously studied tetracyclines (Jones & Morrison, 1963). The slopes of these linear plots, for all five tetracyclines inhibiting the rate of growth by Mode II, in turn plot linearly against the rates of increase of inhibition of rate of growth by Mode II (Fig. 5). Thus

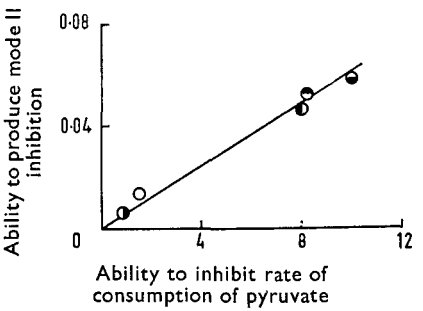


FIG. 5. Relation between mode II inhibition (change of index ratio per mμM antibiotic in basic media at pH 7·0) and inhibition of consumption of pyruvate [min/mM (pyruvate) per 0·1 g (dry-wt) cells/ml per mμM antibiotic]. Antibiotic: tetracycline, ○; oxytetracycline, ◑; 7-chlortetracycline, ◐; 6-demethyl-7-chlortetracycline, ◓; 6-methylene-oxytetracycline, ◒.

the ability of a tetracycline to inhibit the consumption of pyruvate in unaerated medium is quantitatively related to its ability to inhibit rate of growth by Mode II. The simplest explanation is that inhibition of pryuvate consumption is a consequence of Mode II. 6-Demethyl-6-deoxytetracycline, with an ability to affect pyruvate consumption of 0·6 in the units of Fig. 5, should have the ability to inhibit rate of growth by Mode II to an extent of 0·004 per mμM; its ability to inhibit by Mode I is greater than this and hence inhibition of rate of growth is by Mode I.

Discussion

A summary of the relevant literature up to 1960 (Snell & Cheng, 1962) shows the diversity of the interferences with the metabolisms of bacterial cells that can be due to tetracyclines. Tetracyclines have since been found to affect the permeability of the cell walls to amino-acids (Okamoto & Mizuno, 1964) and the transfer of amino-acid to the ribosomal protein in both animal and bacterial systems (e.g. using leucine; Franklin, 1963); the fraction of the ribosomal protein concerned is insoluble in cold trichloracetic acid and the transfer process is dependent on the presence of phospho-enol-pyruvate and another fraction of the ribosome (Franklin, 1964). "The problem becomes one of sorting out their various contributions to inhibition and the order in which they apply (as a function of increasing concentration) to various biological systems" (Snell & Cheng, 1962).

Because they found that 2·1 μM tetracycline (1·0 μg/ml of the hydrochloride), a bactericidal concentration, arrested cell division of *Staphylococcus aureus H* in a medium of undefined pH different to that used in this laboratory, Hash, Wishnik & Miller (1964) suggest erroneously that the optical measurements made on cultures of *Aerobacter aerogenes* in the glucose-salt medium containing 0–0·35 μM tetracycline, have "nothing to do with mean generation times" [*sic*]. Direct measurements of the dry weight of bacterial substance per ml in fully grown cultures confirm that the concentrations of antibiotic used by Jones & Morrison (1962) and by the present authors do not alter significantly the amount of growth produced, and this is correctly obtained from the optical measurements. The mean generation time properly required for the calculation of Index Ratios is the time for the doubling of bacterial mass, viz., log 2 {dt/dlog (dry-wt cells/ml)} and not log 2 {dt/dlog (number of cells/ml)} as was inferred; it was appreciated that any change in size of the bacteria would introduce an error in the latter, which was avoided. In fact, the error would not have been serious; direct measurements with a Coulter medical counter Model A show that all the cultures had an approximately hundred-fold increase in the number of cells per ml and that a change of size could not be detected unless the concentration of antibiotic is near the limit of that required. Hash & others (1964) also do not distinguish between the effects on rate of growth and on amount of growth.

The present use of a method which measures inhibition of growing cells and identifies the mechanism of inhibition, has confirmed with four more tetracyclines the conclusion of Jones & Morrison (1962) that inhibition by a tetracycline is quantitatively related to the concentration of its molecular form in the medium. The two originally described modes of inhibition have again been found to operate but, in addition, a third mode shown by tetracyclines containing a chlorine atom in the 7-position has been identified. The slopes of the plots of Index Ratio against concentration of molecular form provide a measure of the relative intrinsic activities of the various tetracyclines from which the actual activity at a particular pH and concentration of free Mg^{2+}ions, can be calculated (Table 5). The ability

TABLE 5. POTENTIAL ACTIVITIES OF SOME TETRACYCLINES

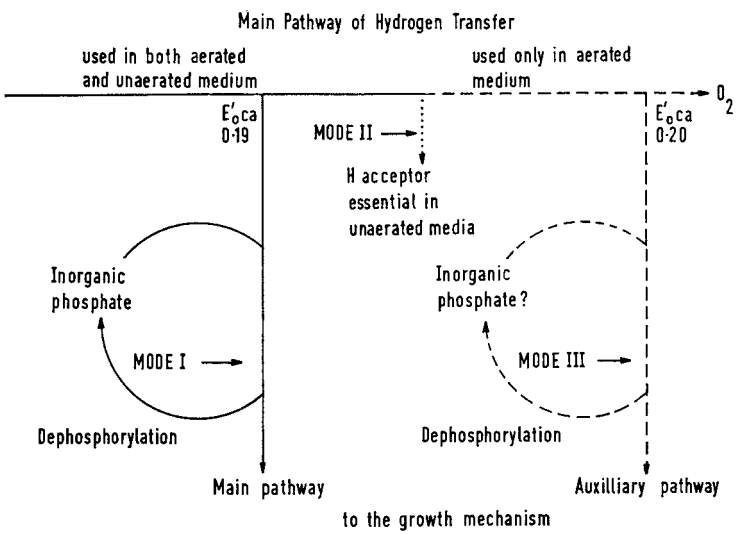
Antibiotic	Change of index ratio per mμM antibiotic in the molecular form					
	Mode I		Mode II		Mode III	
	actual	relative	actual	relative	actual	relative
6-Demethyl-6-deoxytetracycline	0·007	1·0	0·004*	0·5*	?	?
Oxytetracycline	0·007	1·0	0·009	1·2	?	?
Tetracycline	0·007	1·0	0·017	2·2	?	?
6-Methyleneoxytetracycline	0·026	3·6	0·072	9·9	?	?
7-Chlortetracycline	0·028	3·8	0·063	8·6	0·031	4·2
6-Demethyl-7-chlortetracycline	0·026	3·6	0·080	11·0	0·038	5·2

*Calculated from inhibition of consumption of pyruvate.

to inhibit by Mode III may be common to all the tetracyclines but, if so, the intrinsic activity as inhibitors by Mode III of the tetracyclines not containing chlorine at the 7-position, is less than 0·007 per mμM, and inhibition by

Mode I prevents inhibition of rate of growth of *Aerobacter aerogenes* by Mode III. The intrinsic activity to inhibit by Mode I is unaltered by substitution of —OH for —H at the 5-position, and by simultaneous demethylation and replacement of —OH by —H (and probably to demethylation alone) at the 6-position, but it is increased markedly by a methylene group at the 6-position or a chlorine atom at the 7-position. The intrinsic activity as an inhibitor by Mode II is decreased by —OH at the 5-, and by —H replacing —OH at the 6-, but is increased by methylene at the 6- or chlorine at the 7-position. There is no obvious relationship between the intrinsic activities as inhibitors by Modes I and II, and the sizes of the dissociation constants or of the association constants for the complexes with Mg^{2+}. It should be pointed out that these constants do affect the degree of inhibition in practice as that is dependent on the environmental conditions so that, e.g. in our "basic" test medium at pH 7·0, 6-methyleneoxytetracycline is the most potent by Mode II.

Modes I and III, according to the results with added redox systems, measurements of oxygen uptake, and measurements of the initial rates of uptake of inorganic phosphate, are interferences with reaction sequences linking the main pathway of hydrogen transfer from glucose to oxygen, to the growth mechanism. A possible relationship between these two modes and Mode II is:



Reversibility of the reaction sequence in which Mode I occurs, coupled with a release of inorganic phosphate by dephosphorylation of intermediates occurring further down the same reaction sequence, could account for the temporary stimulation of uptake of phosphate that was observed in the presence of tetracycline.

Correlating the present findings with the inhibitions found by other workers is complicated. (*a*) The quantitative relationship of bacteriostatic action to inhibitions of isolated parts of various metabolic systems

in media of differing composition and pH, cannot be assessed without knowing the relationships between internal and external concentrations of materials which are present within the cells and in the surrounding media. (*b*) It may not be possible to associate a published measure of bacteriostatic ability with a definite mode of action. For example, if the present results for chlortetracycline had been expressed as the concentration needed to inhibit the rate of growth by 50% (corresponding to an Index Ratio of 2·0 in Fig. 1) the inhibition could have been by any of the three modes according to the conditions. In many instances inhibitions are expressed as '% inhibition of growth' with no indication of the nature of the actual measurements. (*c*) In the media only the concentrations of the molecular forms of six different tetracyclines are related to the bacteriostatic action and it is impossible to calculate these concentrations unless information about the interrelations of materials is available.

Qualitatively, however, connections may be suggested. Indeed with inhibitions of reductases, dehydrogenases, oxidases, oxidation or reduction systems, and energy requiring systems, possible connections are obvious. The inhibitions in Franklin's experiments with systems isolated from *Escherichia coli* and rat liver cells (1963, 1964) may also be connected with Mode I or Mode II. He used 400 μM chlortetracycline at pH 7·80 in the presence of 10 mM magnesium which, if all the magnesium is assumed to be free, provides 1 μM of the molecular form. Such a concentration is ten times that needed to produce an Index Ratio of 4 by Mode II and eight times that needed to produce the same degree of inhibition by Mode I for *Aerobacter aerogenes*; but the inhibition of the isolated systems may well occur at lower concentrations of antibiotic, and a higher internal pH than that of the medium may result in a higher internal concentration of antibiotic within the intact cells of *Aerobacter aerogenes*. Mode I in *Aerobacter aerogenes* is a derangement of protein synthesis, and though Mode II does not operate in this strain in aerated medium, Franklin's experiments used only part of the whole cell mechanism and he found that phosphoenol-pyruvate was required: there is a connection between Mode II and inhibition of the consumption of pyruvate.

The inhibition of the utilisation of D-glutamate by *Escherichia coli*, which is possibly due to competition between the A-ring of (oxy)tetracycline and D-glutamate for the same enzyme (Snell & Cheng, 1962), might be identified with Mode III which is certainly concerned with D-glutamate. This in turn, as D-glutamate is a constituent of cell walls, could affect the permeability of the walls of *Escherichia coli* as reported for tetracycline (Okamoto & Mizuno, 1964) and chlortetracycline (Alexander, 1960).

Since the kinetics of the investigated inhibitions of intact cells that we report could be dominated by the external concentration of the molecular form of the inhibitor, if that were the only form able to penetrate the cells, it is not possible to rule out mechanisms of inhibition which depend on the power of tetracyclines to form complexes with certain cations. If the kinetics are so dominated, and other forms are inhibitory, the intrinsic activities in Table 5 would each have to be amended by the appropriate factor calculated from the constants in Table 1 and the internal pH

pertaining when the experimental measurements were made. This possibility will be examined later.

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