THE BACTERIOSTATIC ACTIONS OF TETRACYCLINE AND OXYTETRACYCLINE

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The bacteriostatic actions of tetracycline and oxytetracycline on cultures of *A. aerogenes* in glucose-mineral salt media have been studied. Both antibiotics show two modes of action, one of which is non-operative under aerobic conditions and is ascribed to an interference with the disposal of hydrogen in unaerated cultures. The other mode of action can be accounted for by an interference with the synthesis of protein. Quantitative measurements of the dependence of the degree of inhibition on the pH and the concentration of magnesium ions are in accord with attributing inhibitory power solely to the non-ionic molecular forms of the antibiotics. The possible contributions of the two modes of action to the cytotoxic action are discussed.

IT has been suggested variously (Eagle and Saz, 1955) that tetracyclines affect oxidation and fermentation as well as derange the synthesis of protein in cells, and, since they have an affinity for inorganic cations, that they may interfere with cellular and enzymatic processes which require such ions. Many of the investigations which have led to these suggestions have been made with much larger concentrations than those obtained clinically (Goldberg, 1959a) which in the case of tetracycline itself is 1 to 2 μM in the blood (Welch, Lewis, Staff and Wright, 1957), and many were made with isolated parts of bacterial metabolism. Thus, the cytotoxic action of tetracyclines remains obscure.

Recently it has been shown that the ratio of the mean generation time of an inhibited culture to the mean generation time of a corresponding uninhibited culture, which has been termed the "index ratio", is a sensitive and reproducible measure of the amount of inhibition caused by a small concentration of an inhibitor in cultures of a thoroughly stabilised organism, and that abrupt changes in the plot of index ratio against concentration of inhibitor indicate changes from one mode of inhibition to another (Harris and Morrison, 1961). This means of measuring inhibition is now used to show that removal of inorganic cations is not a likely mode of action of tetracycline or oxytetracycline, to establish that only one of the possible molecular and ionic forms (Goldberg, 1959b) has inhibitory powers, and to show that both tetracyclines have two modes of action at concentrations that are realisable clinically.

METHODS AND MATERIALS

Organism and Media

Since the method requires a thoroughly stabilised organism which possesses the maximum synthesising abilities two sub-strains of the *Aerobacter aerogenes* utilised by Dagley, Dawes and Morrison (1951),

which had been maintained by regular sub-culturing (unaerated and aerated respectively) in synthetic medium for eight years, were selected. Both sub-strains showed the same reactions to various sugars in Bacto-purple broth, to Bacto Simmons Citrate Agar, MR-VP medium, and Bacto-SIM medium (except for motility in the latter test) as described for *A. aerogenes* in the 9th edition of the Difco manual. The basic medium contained 5·4 g. KH_2PO_4, 12 g. glucose, 1·8 g. $(NH_4)_2SO_4$ and 0·0203 g. $MgSO_4 \cdot 7H_2O$ (all Analar quality) per litre of demineralised water of resistance greater than 2×10^6 ohms per cm., and was adjusted to the required pH (usually 7·00) with NaOH. The concentration of magnesium in this medium is 0·082 mM which is one tenth of the lower limit of the concentration (2 to 4 mg./100 ml.) in human blood (Bell, Davidson and Scarborough, 1950), but twice the minimum concentration required to maintain the normal growth characteristics of the test organism. When higher concentrations of magnesium or the presence of other materials were required, part of the water in the basic medium was replaced by a suitable solution. All inocula (usually 0·10 ml. to 10·0 ml. of medium) were taken after growth had stopped, from parent cultures containing only sufficient glucose to permit two-thirds of the normal extent of growth; a procedure which ensures reproducible results and negligible lags before growth. The extents of growth were obtained by measurements of optical density of killed samples using a "Unicam S.P.400" spectrophotometer at 583 mμ calibrated in mg. dry cells per ml. Rates of growth (mean generation times) were obtained graphically, were reproducible to ± 3 per cent, and could be determined from data obtained from a growing culture before its pH had fallen by 0·2 units. Cultures in unaerated basic media in 7 in. by 1 in. tubes of the sub-strain accustomed to the conditions, have a mean generation time of 38 ± 1 min., and cultures of the other sub-strain in basic media aerated with sterile, filtered air containing an adequate concentration of CO_2 (Morrison, Griffiths and Harris, 1955), have a mean generation time of 32 ± 1 min.

Antibiotics

Tetracycline as the hydrochloride "Tetracyn" and oxytetracycline as "Terramycin" were donated generously by Messrs. Pfizer Ltd. Aqueous solutions of each, and solutions containing magnesium ions, were titrated potentiometrically with aqueous NaOH using the conditions described by Albert and Rees (1956). The dissociation constants K_1, K_2 and K_3, and the stability constants K_S for the complexes with magnesium, calculated conventionally, are given with corresponding values from the literature in Table I.

Since each compound has four ionic or molecular forms $TH_3^+Cl^-$ will be used to represent tetracyline hydrochloride and $\emptyset H_3^+Cl^-$ to represent oxytetracycline hydrochloride.

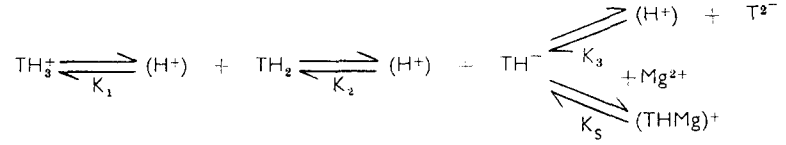
The potentiometric titration curves in the presence and absence of magnesium are concurrent initially when, by calculation from the dissociation constants, the tetracycline compound is present as a mixture of TH_3^+ and TH_2, or $\emptyset H_3^+$ and $\emptyset H_2$, but deviate when it is present as a

TABLE I

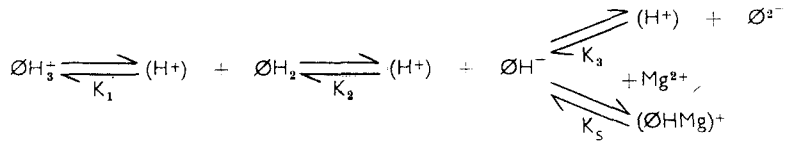
IONISATION CONSTANTS (K_1, K_2, K_3) OF TETRACYCLINE AND OXYTETRACYCLINE HYDROCHLORIDES AND STABILITY CONSTANTS K(S) OF THE COMPLEXES WITH MAGNESIUM

Source of information	Tetracycline				Oxytetracycline			
	pK_1	pK_2	pK_3	pK_S	pK_1	pK_2	pK_3	pK_S
Present work	3·45	7·92	9·81	4·16–4·29	3·46	7·76	9·70	3·96
Albert (1953) or Albert and Rees (1956)	3·35	7·82	9·57		3·10	7·26	9·11	3·80
Stephens and others (1956)	3·30	7·68	9·69		3·27	7·32	9·11	
Regna and others (1951) ..					3·45	7·55	9·24	

mixture of TH_2 and TH^-, or $\emptyset H_2$ and $\emptyset H^-$. Clearly Mg^{2+} combines with the ionic forms TH^- and $\emptyset H^-$. Between pH 7·00 and 7·80 the forms of the compounds present in significant concentrations are TH_2 and TH^-, and $\emptyset H_2$ and $\emptyset H^-$. The relationship between the molecular and ionic forms are:



and



RESULTS

Extent of Inhibition and Concentration of Antibiotic

In basic media. Increasing concentrations of either antibiotic cause a progressive increase of mean generation time of both aerated and unaerated cultures. The results of plotting the calculated index ratios against the molar concentrations of antibiotic are shown in Fig. 1.

After a threshold concentration of between 0 and 0·035 μM tetracycline, in aerated cultures, and in unaerated cultures up to 0·2 μM, the index ratios are found to plot together (Mode I tetracycline). At concentrations greater than 0·2 μM in unaerated cultures tetracycline inhibits more severely (Mode II tetracycline). Inhibition by oxytetracycline of aerated cultures (Mode I oxytetracycline) resembles closely Mode I tetracycline, but the inhibition of unaerated cultures is more severe at all concentrations (Mode II oxytetracycline).

In enriched media. Adding to the basic medium the product of an inhibited reaction, provided that it can penetrate the cells, must eliminate the inhibited reaction's control of the rate of growth (Harris and Morrison, 1961). The following materials were used to enrich the medium: bacteriological peptone (Difco), casein hydrolysate (prepared by D. E. Griffiths in this laboratory and consisting solely of amino-acids), individual

amino-acids (Messrs. Light or B.D.H.) and yeast-extract (Difco), all of which can replace the glucose or the ammonium salt, or both, in basic medium without preventing growth of the organism. None of these materials affected the degree of inhibition of aerated cultures by 0 to

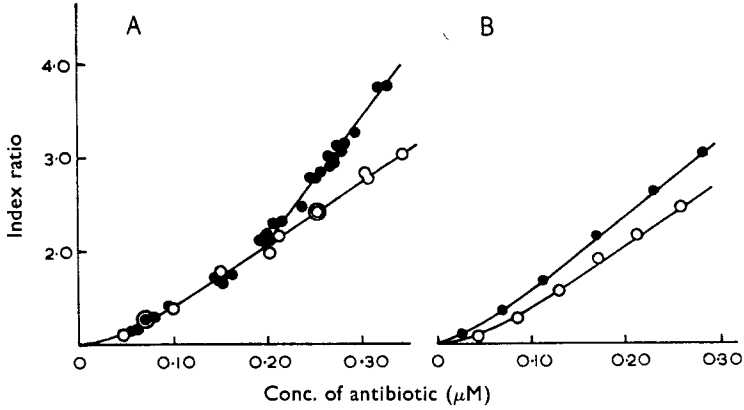


FIG. 1. Inhibition of cultures of *A. aerogenes* by tetracycline and oxytetracycline. Index ratio is (the mean generation time of the inhibited culture) divided by (the mean generation time of the uninhibited culture). A, tetracycline; B, oxytetracycline; ●, unaerated cultures; ○, aerated cultures.

0·35 μM tetracycline or oxytetracycline, nor did the peptone, casein hydrolysate nor any individual amino-acid affect the inhibition of unaerated cultures. The affect of yeast-extract at concentrations of 0·2 per cent w/v or greater in unaerated cultures are compared with the inhibitions of plain unaerated and aerated cultures in Table II.

In both examples the inhibition of unaerated cultures containing yeast-extract is indistinguishable from the inhibition of aerated cultures in basic

TABLE II

EFFECT OF 0·2 PER CENT W/V YEAST-EXTRACT ON INHIBITION BY TETRACYCLINE AND OXYTETRACYCLINE

Concentration of antibiotic μM	Index ratio ±0·08		
	A Unaerated basic medium	B A plus yeast-extract	C Aerated basic medium
Tetracycline			
0·051	1·12	1·12	1·14
0·103	1·40	1·37	1·40
0·154	1·68	1·67	1·75
0·206	2·15	2·03	2·10
0·214	2·22	2·07	2·16
0·220	2·30	2·05	2·20
0·248	2·70	2·31	2·40
0·258	2·80	2·36	2·46
0·275	3·10	2·59	2·58
0·283	3·05	2·61	2·64
0·303	3·47	2·83	2·77
Oxytetracycline			
1·02	1·56	1·40	1·36
2·26	2·62	2·40	2·22
3·19	3·30	2·88	2·92

medium. Thus yeast-extract provides an "antidote" to both Mode II tetracycline and to Mode II oxytetracycline.

The nucleotide fraction of the yeast-extract isolated by the method of Clark, Dounce and Stotz (1949) did not reproduce the effect of the yeast-extract on inhibition by tetracycline. In further tests the dicarboxylic acids oxalo-acetic, fumaric and succinic were used: none decreased the inhibition of aerated cultures by tetracycline and only fumaric at 0·4 per cent w/v had a detectable effect on inhibition of unaerated cultures but this is not sufficient to be an elimination of Mode II tetracycline (Table III).

TABLE III

EFFECT OF 0·4 PER CENT W/V FUMARIC ACID ON INHIBITION OF UNAERATED CULTURES BY TETRACYCLINE

Concentration of tetracycline, μM	Index ratio ±0·08		
	A Unaerated basic medium	B A plus fumarate	C Aerated basic medium
0·214	2·22	2·07	2·16
0·299	3·44	3·20	2·75
0·320	3·72	3·47	2·89

These results suggest that the efficiency of the yeast-extract is due to its content of reducible substance(s) and not to its provision of co-enzymes I or II.

Extent of Inhibition and the Concentration of Magnesium

Tetracycline in unaerated cultures. The index ratios were determined for cultures containing varying concentrations of magnesium (82 μM to 6·56 mM) and tetracycline (0 to 0·3 μM). In all experiments the angles, measured from the horizontal ordinate, of the plots corresponding to those in Fig. 1, decrease progressively as the concentration of magnesium increases. This indicates a decreasing degree of inhibition. The results for unaerated cultures inhibited by tetracycline are shown in Fig. 2.

Since magnesium ions associate with sulphate and phosphate ions the concentration of free magnesium ions must be calculated before the experimental data can be examined to ascertain whether the inhibitors operate by removing magnesium ions or whether magnesium ions decrease the inhibition by removing molecules or ions of the inhibitor.

The medium contains 0·0397 F KH_2PO_4 and 0·0136 F $(NH_4)_2SO_4$ and both phosphate and sulphate associate with Mg^{2+} ions.

Since pK_2 of $H_2PO_4^-$ is 7·2 at 25° (Robinson and Stokes, 1959) at pH = 7·00, $[HPO_4^{2-}] = 0{\cdot}0153$ and $[HPO_4^{2-}]/[H_2PO_4^-] = 0{\cdot}63$ in the presence of Mg^{2+} ions,

$$[HPO_4^{2-}] = C_L - [H_2PO_4^-] - [MgHPO_4]$$

where C_L = the total concentration of phosphate = 0·0379, and assuming that Mg^{2+} and $H_2PO_4^-$ do not associate,

$$\therefore [C_L - [H_2PO_4^-] - [MgHPO_4]]/[H_2PO_4^-] = 0{\cdot}63 \quad \ldots \quad \ldots \quad (1)$$

Since K of HSO_4^- = 0·01 (Robinson and Stokes, 1959), $[HSO_4^-]$ is negligible.

Now (Dunsmore and James, 1951):

$$K_{s(MgSO_4)} = [Mg^{2+}]\,[SO^{2-}]/[MgSO_4] = 0{\cdot}006$$

and (Redish and Kibrick, 1940)

$$K_{s(MgHPO_4)} = [Mg^{2+}]\,[HPO_4^{2-}]/[MgHPO_4] = 0{\cdot}003$$

Hence

$$[MgHPO_4] = [C_M - [MgHPO_4] - [MgSO_4]]\,[HPO^{2-}]/0{\cdot}003 \qquad (2)$$

and

$$[MgSO_4] = [C_M - [MgHPO_4] - [MgSO_4]]\,[0{\cdot}0138 - [MgSO_4]]/0{\cdot}006 \qquad (3)$$

where C_M = the total concentration of magnesium, which in the basic medium is 82 μM.

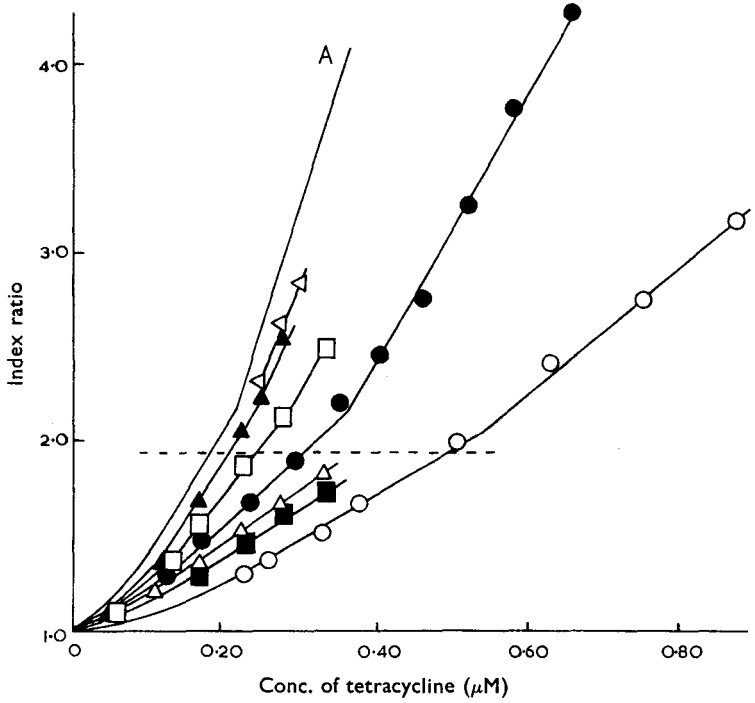


FIG. 2. Magnesium concentration and inhibition of unaerated cultures of *A. aerogenes* by tetracycline. Concentration of magnesium (mM): A, from Fig. 1, 0·082; ◁, 0·656; ▲, 0·820; □, 1·64; ●, 2·46; △, 3·28; ■, 4·10; ○, 6·56.

Ignoring $[MgSO_4]$ in the right hand side of equation (2), from (1) and (2) $[MgHPO_4] \doteqdot 68$ μM and $[Mg^{2+}] \doteqdot 1{\cdot}4$ μM. Putting these rough values into (3) $[MgSO_4) \doteqdot 32$ μM. Since $C_M = [Mg^{2+}] + [MgHPO_4] + [MgSO_4]$ the rough value for $[Mg^{2+}]$ is too high, but trial and error with

lower values calculating $[MgHPO_4]$ and $[MgSO_4]$ to test in the equation for C_M establishes that

$$[Mg^{2+}] = 9{\cdot}8\ \mu M \text{ and } \therefore [Mg^{2+}]/C_M = 0{\cdot}120$$

Similarly when $C_M = 6{\cdot}56$ mM, $[Mg^{2+}]/C_M = 0{\cdot}132$.

Thus in all the media at pH 7·00 only $\frac{1}{8}$th of the magnesium was present as free Mg^{2+} ions after allowing for association with phosphate and sulphate. In the present experiments the concentrations of inhibitors varied from 0 to 1 μM whereas the concentration of free Mg^{2+} ions varied from 10·2 to 86·6 μM. Association of Mg^{2+} and tetracycline or oxytetracycline cannot have had a significant effect on the concentration of free Mg^{2+} ions and hence neither antibiotic can be exerting an inhibitory action by decreasing the concentration of free Mg^{2+} ions. Since the values of K_s for the interactions of other cations with the two tetracyclines are similar to that for the magnesium complexes (Albert and Rees, 1956) and other cations must be assumed to associate with sulphate and phosphate, it is unlikely that the inhibitory actions can be accounted for by a decrease in the concentration of any other cation in the presence of the antibiotics.

If the higher concentrations of magnesium decrease the inhibition by decreasing the concentration of free antibiotic then points having the same index ratio on the experimental plots, one for each total concentration of magnesium, in Fig. 2 (for example intercepts with the horizontal broken line) must correspond to cases in which the concentration of free antibiotic is the same. It is thus possible to obtain a number of series of paired concentrations of magnesium and of an antibiotic which yield the same concentration of free antibiotic. The plot of concentration of magnesium against concentration of antibiotic for each such series is a shallow curve which can be extrapolated shortly to cut the concentration of antibiotic ordinate. Each intercept gives the concentration of antibiotic which would cause the particular degree of inhibition in the absence of association with magnesium, and hence the concentration of free antibiotic responsible for the particular value of the index ratio for the series.

Since at pH 7·00 the free tetracycline is present solely as TH_2 and TH^-, and the dissociation constant K_2 is known, the concentrations of TH_2 and TH^- can be calculated from the concentration of free tetracycline. It is thus possible to obtain corresponding values of $[TH_2]$, $[TH^-]$, [bound tetracycline] (total concentration of tetracycline $- [TH_2] - [TH^-]$) and [free Mg^{2+}] (calculated from total concentration of magnesium as before) provided that the total concentration of magnesium is sufficiently large to ensure that $[Mg^{2+}$ (free)] is not affected significantly by combination of magnesium and tetracycline.

When the experimental data used for Fig. 2 are processed in this way, for any one particular index ratio log $[Mg^{2+}]$ plots linearly with a slope of unity against log [bound tetracycline], and log $[TH^-]$ also plots linearly with a slope of unity against the same ordinate except when the concentration of tetracycline is relatively high, then the slope decreases. This indicates that the complex between magnesium and tetracycline is

normally $MgTH^+$ but possibly some $Mg(TH)_2$ is obtained at the higher concentrations of tetracycline. The previously determined [bound tetracycline] may be expressed as $[MgTH^+]$.

Since the stability constant, K_s, for $MgTH^+$ is given by

$$\log K_s = \log [MgTH^+] - \log [Mg^{2+}] - \log [TH^-]$$

$\log K_s$ can be calculated from the results of the experiments with bacteria. This is done in Tables IV and V.

TABLE IV

CALCULATION OF LOG K_s FOR THE MAGNESIUM-TETRACYCLINE COMPLEX. PART 1

Index Ratio	T_f	[Mgt] $[Mg^{2+}]$	82·0 10·25	164 20·50	246 30·75	328 41·00	410 51·25	656 82·00
1·20	0·64– 0·69	T_t	0·78	0·85	1·00	1·16	1·35	1·66
		T_b	0·09– 0·14	0·16– 0·21	0·31– 0·36	0·47– 0·52	0·66– 0·71	0·97– 1·02
1·40	0·97– 1·00	T_t	1·17	1·29	1·61	1·79	2·13	2·58
		T_b	0·17– 0·20	0·29– 0·32	0·61– 0·64	0·79– 0·82	1·13– 1·16	1·58– 1·61
1·60	1·29	T_t	1·50	1·72	2·14	2·54	2·90	3·40
		T_b	0·21	0·43	0·85	1·25	1·61	2·11
1·80	1·58	T_t	1·83	2·13	2·68	3·22	3·65	4·18
		T_b	0·25	0·55	1·10	1·64	2·07	2·60
2·00	1·85	T_t	2·14	2·56	3·22			4·97
		T_b	0·29	0·71	1·37			3·12
2·20	2·09	T_t	2·42	2·92	3·64			5·64
		T_b	0·33	0·83	1·55			3·55
2·40	2·23	T_t	2·64	3·18	3·94			6·30
		T_b	0·41	0·95	1·71			4·07
2·60	2·38	T_t			4·23			6·94
		T_b			1·85			4·56
2·80	2·54	T_t			4·52			7·57
		T_b			1·98			5·03
3·00	2·69	T_t			4·81			8·19
		T_b			2·12			5·50
3·20	2·83	T_t			5·09			8·80
		T_b			2·26			5·97

T_t = Total concentration of tetracycline ($M \times 10^7$)
T_f = Concentration of free tetracycline ($M \times 10^7$), $Tb = Tt - Tf$.
[Mgt] = Total concentration of magnesium $(M \times 10)^2$
$[Mg^{2+}]$ = Concentration of Mg^{2+} ions ($M \times 10^6$)

Thus the theory of a non-inhibitory complex of magnesium and tetracycline requires the complex to be $MgTH^+$ with a stability constant of antilog (4·07 to 4·38) in order to have a quantitative fit to the experimental evidence. Potentiometric titrations require the formula to be $MgTH^+$ and the stability constant to be antilog (4·16 to 4·29) (Table I).

Extent of inhibition and pH

Tetracycline in unaerated cultures. The overall appearance of the graphical representations corresponding to Fig. 1 of the results of experiments in media having an initial value of pH other than 7·00, is unchanged, but decreasing the pH increases the slopes of the linear portions of the figure and increasing the pH decreases them. The index ratios for a number of concentrations of tetracycline in unaerated cultures in basic media initially at pH 7·00 and 7·80 are compared in Table VI.

Since at pH 7·00 and 7·80 the tetracycline is present as TH_2, TH^- and $MgTH^+$, and it has been concluded that the latter is non-inhibitory, both modes of action must be due to TH_2 and/or TH^-. At higher pH

TABLE V

CALCULATION OF LOG K_S FOR THE MAGNESIUM-TETRACYCLINE COMPLEX. PART 2

log $[Mg^{2+}]$	$\bar{4}\cdot01$		$\bar{4}\cdot31$		$\bar{4}\cdot49$		$\bar{4}\cdot62$		$\bar{4}\cdot71$		$\bar{4}\cdot92$	
log $[TH^-]$	log T_b	log K_S	log T_b	log K_S	log T_b	log K_S	log T_b	log K_S	log T_b	log K_S	log T_b	log K_S
$\bar{9}\cdot87$ or $\bar{9}\cdot84$	$\bar{9}\cdot95$	4·07	$\bar{8}\cdot20$	4·07	$\bar{8}\cdot49$	4·13	$\bar{8}\cdot67$	4·18	$\bar{8}\cdot82$	4·24	$\bar{8}\cdot99$	4·20
$\bar{8}\cdot15$	$\bar{8}\cdot15$	4·31	$\bar{8}\cdot32$	4·17	$\bar{8}\cdot56$	4·23	$\bar{8}\cdot72$	4·26	$\bar{8}\cdot85$	4·30	$\bar{7}\cdot01$	4·25
$\bar{8}\cdot03$ or $\bar{8}\cdot02$	$\bar{8}\cdot23$	4·19	$\bar{8}\cdot46$	4·12	$\bar{8}\cdot79$	4·27	$\bar{8}\cdot90$	4·25	$\bar{7}\cdot05$	4·31	$\bar{7}\cdot20$	4·25
$\bar{8}\cdot30$	$\bar{8}\cdot30$	4·27	$\bar{8}\cdot51$	4·18	$\bar{8}\cdot81$	4·30	$\bar{8}\cdot91$	4·27	$\bar{7}\cdot06$	4·33	$\bar{7}\cdot21$	4·27
$\bar{8}\cdot14$	$\bar{8}\cdot32$	4·17	$\bar{8}\cdot63$	4·18	$\bar{8}\cdot93$	4·30	$\bar{7}\cdot10$	4·34	$\bar{7}\cdot21$	4·36	$\bar{7}\cdot32$	4·25
$\bar{8}\cdot23$	$\bar{8}\cdot40$	4·16	$\bar{8}\cdot74$	4·20	$\bar{7}\cdot04$	4·32	$\bar{7}\cdot21$	4·36	$\bar{7}\cdot32$	4·38	$\bar{7}\cdot42$	4·27
$\bar{8}\cdot30$	$\bar{8}\cdot46$	4·15	$\bar{8}\cdot85$	4·24	$\bar{7}\cdot14$	4·35					$\bar{7}\cdot49$	4·27
$\bar{8}\cdot35$	$\bar{8}\cdot52$	4·16	$\bar{8}\cdot92$	4·26	$\bar{7}\cdot19$	4·35					$\bar{7}\cdot55$	4·28
$\bar{8}\cdot38$	$\bar{8}\cdot61$	4·22	$\bar{8}\cdot98$	4·29	$\bar{7}\cdot23$	4·36					$\bar{7}\cdot61$	4·31
$\bar{8}\cdot41$					$\bar{7}\cdot27$	4·37					$\bar{7}\cdot66$	4·33
$\bar{8}\cdot43$					$\bar{7}\cdot30$	4·38					$\bar{7}\cdot70$	4·35
$\bar{8}\cdot46$					$\bar{7}\cdot33$	4·36					$\bar{7}\cdot74$	4·36
$\bar{8}\cdot48$					$\bar{7}\cdot35$	4·38					$\bar{7}\cdot78$	4·38

$\log K_S = \log Tb - \log [Mg^{2+}] - \log [TH^-] = 4{\cdot}07$ to $4{\cdot}38$

the $[TH^-]$ due to a particular concentration of tetracycline is larger, $[TH_2]$ is smaller and the inhibitory power is less. This suggests that TH_2 is the inhibitor, in which case the degree of inhibition should reflect $[TH_2]$ irrespective of the pH.

From the equations

$$K_S = [MgTH^+]/[Mg^{2+}]\,[TH^-]$$

$$K_2 = [H^+]\,[TH^-]/[TH_2]$$

$$T_t = [TH^-] + [TH_2] + [MgTH^+]$$

where T_t = the total concentration of tetracycline,

$$\text{at pH } 7{\cdot}00\ [TH_2] = T_t\,(8{\cdot}32/9{\cdot}53)$$

$$\text{at pH } 7{\cdot}80\ [TH_2] = T_t\,(1{\cdot}32/2{\cdot}53)$$

In Fig. 3A the extent of inhibition in media initially at pH 7·00 and at pH 7·80 is plotted against T_t (broken lines) and against $[TH_2]$ (solid line). Clearly the experimental data fit the theory that of all the molecular and ionic forms only TH_2 is inhibitory.

TABLE VI

EFFECT OF PH ON INHIBITION BY TETRACYCLINE

Concentration of tetracycline (μM)..	0	0·060	0·120	0·180	0·240	0·360	0·480
Index ratio at pH 7·00	1·00	1·17	1·52	1·94	2·58	4·18	
±0·08 at pH 7·80	1·00	1·12	1·30	1·52	1·82	2·62	3·55

Extent of inhibition by oxytetracycline and its dependence on concentration of magnesium and pH. Measurements of inhibition by oxytetracycline of unaerated cultures in media containing varying concentrations of magnesium at initially pH 7·00 and in basic media at initially pH 7·80, are collated in Tables VII and VIII. Increasing the pH or the concentration of magnesium decreases the slopes of the linear portions of the diagrams corresponding to Fig. 1b, and hence attributing inhibition to the

molecular form of oxytetracycline, $\emptyset H_2$, should be tested quantitatively. At pH 7·00 and pH 7·80 the significantly effective equilibria are

$\emptyset H_2 = \emptyset H^- + (H^+)$ for which $K_2 = [H^+]\,[\emptyset H^-]/[\emptyset H_2]$

$\emptyset H^- + Mg^{2+} = Mg\emptyset H^+ \qquad K_s = [Mg\emptyset H^+]/[\emptyset H^-]\,[Mg^{2+}]$

Since $\emptyset_t = [\emptyset H^-] + [\emptyset H_2] + [Mg\emptyset H^+]$ = the total concentration of oxytetracycline,

$$[\emptyset H_2] = \emptyset_t/[1 + K_2[1 + K_s[Mg^{2+}]]/[H^+]]$$

Since $[Mg^{2+}]$ can be calculated from the total concentration as previously, $[\emptyset H_2]$ for each value of $\emptyset_t$ in Tables VII and VIII can be calculated, and

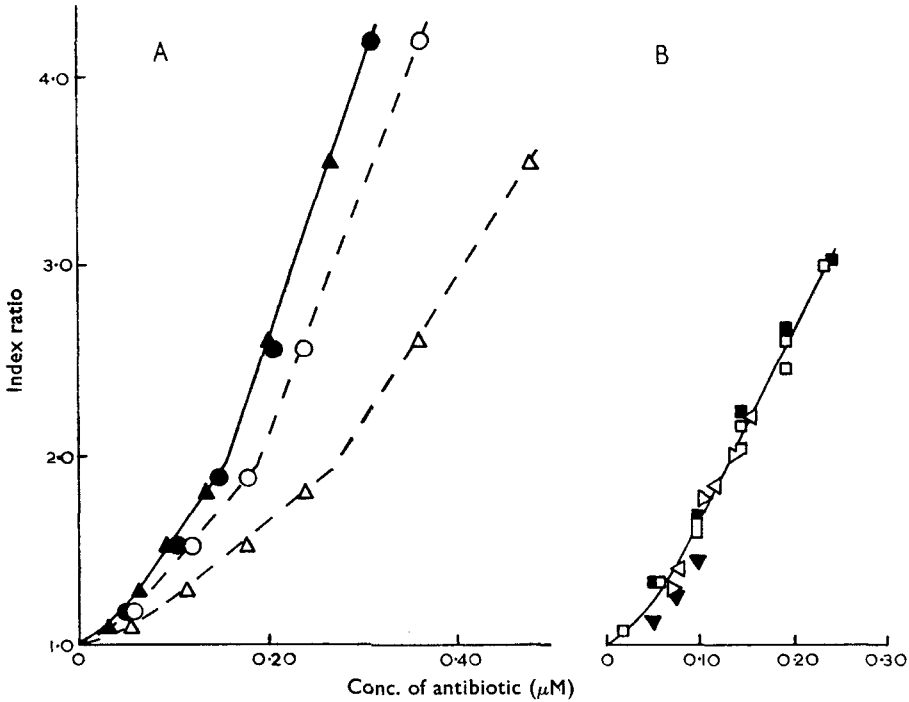


FIG. 3. Inhibition of *A. aerogenes* as a function of the concentrations of the molecular forms of tetracycline and oxytetracycline. Concentrations of molecular tetracycline (TH_2) and of molecular oxytetracycline ($\emptyset H_2$) are calculated using the concentrations of magnesium, sulphate and phosphate, and the initial pH of the unaerated cultures.

	tetracycline (A)		oxytetracycline (B)				
Concentration of magnesium (mM)	0·082	0·082	0·082	0·82	1·64	4·96	0·082
Initial pH	7·00	7·80	7·00	7·00	7·00	7·00	7·80
Symbol	●	▲	□	◁	▷	▼	■

The broken lines, —○—, and —△—, show the plots resulting from using the total concentrations of tetracycline for the cultures initially at pH 7·00 and 7·80, respectively.

used as the ordinate in plots of index ratio against concentration of inhibitor. Fig. 3B shows that the result is a single graph and hence attributing inhibitory power to only $\emptyset H_2$ is in quantitative agreement with the experimental results.

Inhibition in aerated cultures. The experimental results for tetracycline and oxytetracycline inhibiting aerated cultures examined as in the previous paragraph also are in agreement with TH_2 and $\emptyset H_2$ being the inhibitory forms of the substances.

TABLE VII

AFFECT OF MAGNESIUM CONCENTRATION ON INHIBITION OF UNAERATED CULTURES BY OXYTETRACYCLINE

Concentration of oxytetracycline μM	Index ratio ±0·08			
	Concentration of magnesium (mM)			
	0·082	0·820	1·640	4·940
0·107	1·60	1·40	1·30	1·11
0·157	2·02	1·85	1·57	1·25
0·210	1·45	2·23	2·00	1·43

TABLE VIII

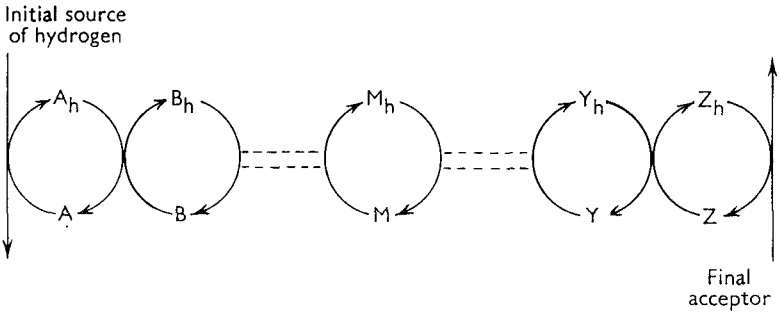
EFFECT OF pH ON INHIBITION OF UNAERATED CULTURES BY OXYTETRACYCLINE

Concentration of tetracycline (μM)	0	0·023	0·068	0·113	0·169	0·226	0·283	0·339	0·452	0·565
Index ratio at pH 7·00	1·00	1·08	1·34	1·66	2·16	2·61	3·02			
±0·08 at pH 7·80	1·00			1·34		1·68		2·24	2·68	3·02

Nature of the Modes of Action

Mode II. With both antibiotics the comparison of the experimental evidence from cultures in basic and in enriched media indicates that mode II is concerned with hydrogen-transfer processes, possibly being inhibition of the production of a hydrogen acceptor required under unaerated conditions.

The transfer of hydrogen in an active bacterial system is effected by a chain of enzyme controlled reactions:



where A_h, B_h etc. are the reduced forms of A, B, etc., respectively. If the enzymic mechanism of such a chain is put in contact with the source of hydrogen, then initially there are no intermediates such as B, M or Y present. The rate of formation of any intermediate is a net rate: it is

being formed from its precursor and being consumed to form its successor, i.e., dS_M/dt = a function, f, of S_L,S_M − a function, f′, of S_M, S_N

$$= f(S_L,S_M) - f'(S_M,S_N)$$

where S_L is the concentration of the precursor, S_M the concentration of intermediate itself, and S_N the concentration of its successor.

The first term increases as S_L increases but decreases as S_M increases and the second increases as S_M increases, but decreases as S_N increases. Thus the net rate of production of the intermediate rises to a maximum and then decreases until $f(S_L,S_M) = f'(S_M,S_N)$ and the steady state is attained with an overall transfer rate for hydrogen of the value of $f(S_L,S_M)$ when $dS_M/dt = 0$. The consequence of this is that a plot of concentration

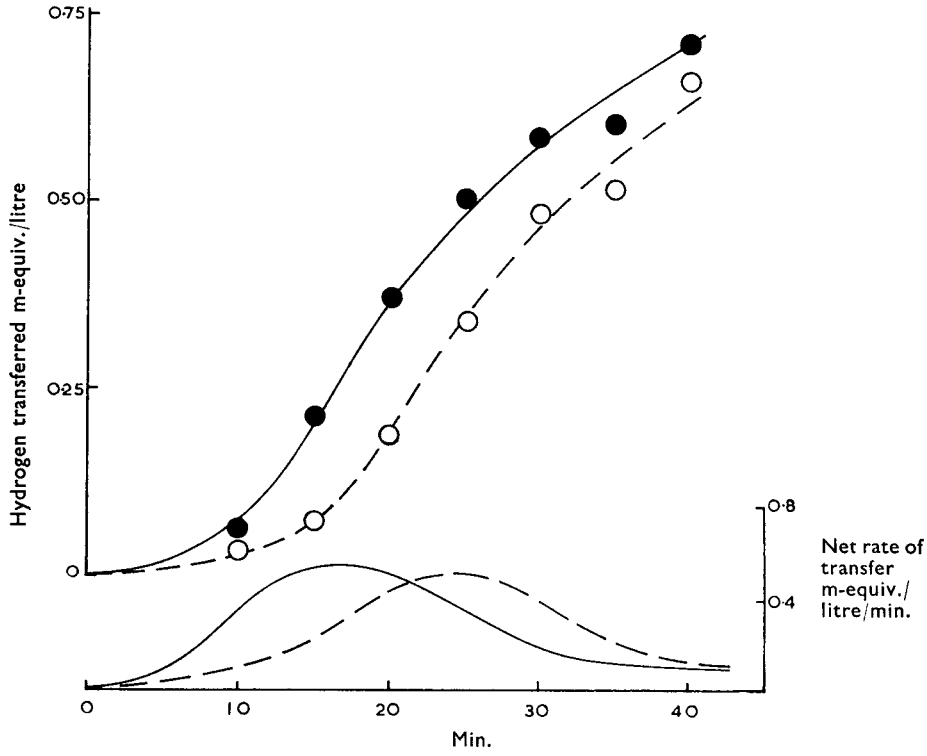


FIG. 4. Effect of tetracycline on the reduction of triphenyltetrazolium ions by a suspension of *A. aerogenes* (0·15 g. (dry) cells per litre). Concentration of tetracycline (μM): ○, 0; ●, 0·30.

of an intermediate against time is a sigmoid curve. The steady state is approached asymptotically and it is not possible to measure the time for it to be reached, but the time at which the maximum net rate of production of the intermediate is reached, is readily determinable as the time of inflexion of the sigmoid curve of concentration. These phenomena are illustrated by the experimental results shown in Fig. 4.

When a colourless substance which is reducible by an intermediate, (say) M_h, to a coloured substance, is added, it forms a non-enzymic branch to the system of which the coloured substance is the end-product. The rate of production of colour is dependent on the rate of production of M_h and hence will rise initially to a maximum and then decrease to a constant value when the enlarged system is in its steady state. This eventual constant rate of production of colour will absorb a definite fraction of the rate of supply of hydrogen to the system and be linearly related to the rate of production of the normal end-product. The time of maximum rate of development of colour is given by the inflexion in the curve of depth of colour against time.

The introduction of an inhibitor of a step preceeding M_h decreases $f(S_L,S_M)$, and of an inhibitor of a step succeeding M_h decreases $f'(S_M,S_N)$. In the former case the time needed for the development of the maximum rate is increased, and in the latter it is decreased. Thus comparison of these times for the inhibited and uninhibited systems indicates whether the inhibition preceeds or succeeds the point of interception of hydrogen by the leuco-compound.

Triphenyltetrazolium ions were reduced at 37° to formazan by suspensions of cells (0·15 g. (dry) cells per litre) in 12 g. glucose, 5·4 g. KH_2PO and 0·0203 g. $MgSO_4 \cdot 7H_2O$ per litre adjusted to pH 7·00, and in medium of the same composition except that 0·3 μmole/litre of tetracycline had been added. This reagent is reduced probably by one of the flavoproteins (Brodie and Gots, 1951; Somerson and Morton, 1953). The cells were obtained from fully grown cultures (unaerated) in which growth had been limited simultaneously by exhaustion of the glucose and ammonium salt originally present, and were resuspended directly in the test media. Cells prepared in this way are potentially highly active but in the absence of a source of hydrogen do not produce a detectable amount of formazan in 1 hr. Well washed cells with the source of hydrogen reduce triphenyltetrazolium ions slowly, presumably because of the loss of co-factors. The extraction and estimation of the formazan was based on the methods of Kun and Abood (1949) and Fahmy and Walsh (1952). Fig. 4 shows that the inflexion point occurs earlier for the inhibited system (16 min. instead of 25) and hence that inhibition by mode II tetracycline can be accounted for by interference with hydrogen transfer at a step subsequent to that at which hydrogen is intercepted by triphenyltetrazolium ions. Similar results were obtained with 0·3 μM oxytetracycline.

The absence of inhibition by mode II in aerated cultures is also explicable. A branch of the transfer system must be capable of transferring hydrogen to oxygen as the final acceptor and only be operative when oxygen is available. If the point at which this branch begins preceeds the reaction inhibited then the inhibition cannot decrease the rate of removal of hydrogen from the initial source.

Mode I. The facts that, with both antibiotics, enriching the medium, including the addition of amino-acids, and aerating the cultures, do not

lessen mode I inhibition, suggests that this interference is with the utilisation of amino-acids for growth, and there are reports that the tetracyclines derange the synthesis of protein (Eagle and Saz, 1955). Such an interference with the metabolism must result in other phenomena and the experimental detection of such phenomena under mode I conditions would be strong support for the suggestion.

During the logarithmic phase of growth of a culture, the metabolism of an organism is in a steady state in which all the metabolic intermediates have a constant concentration determined by the balancing of the combined rates of all reactions producing a particular intermediate and the combined rates of all reactions utilising that intermediate. Any decrease in a rate of utilisation of an intermediate must result in a new equilibrium and a higher steady state concentration of the intermediate.

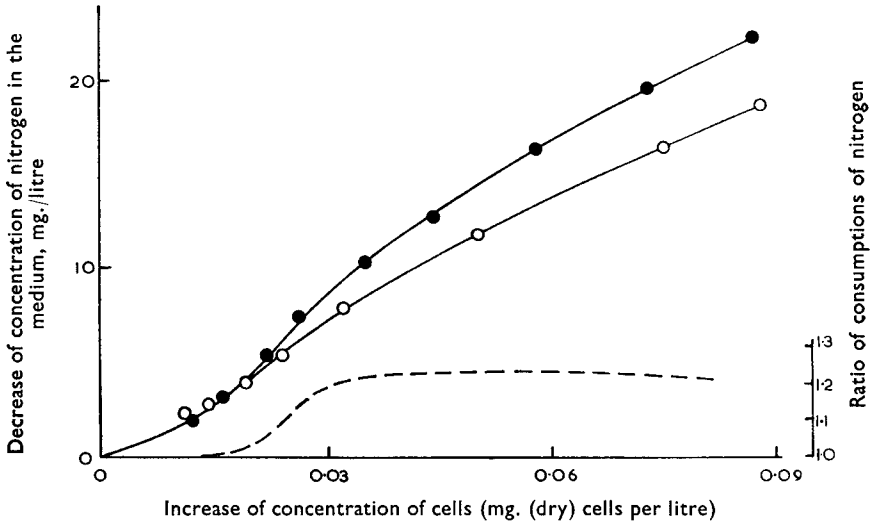


FIG. 5. Effect of tetracycline on the utilisation of nitrogen by *A. aerogenes*. Concentration of tetracycline (μM): ○, 0; ●, 0·10.

Thus a decrease in the rate of utilisation of amino-acids results in a higher concentration of amino-acids. The change, however, must be small since increase of concentration of amino-acids is likely to expedite other utilisations and to depress their own rates of production. The latter in turn causes an increase in concentration of the precursors of the amino-acids. The net result is a period of adjustment during which the intake of substances from the medium is maintained but the concentrations of all intermediates rise and the output of some waste materials may also increase. This period ends when the effect of the initial interference has spread to the whole metabolism and the rate of intake of material from the medium is decreased also and brought into balance with the combined output of cells and waste materials. Measurements of concentrations of any one intermediate or class of intermediate would have to be made with

extraordinary accuracy to detect the change, but during the period of adjustment the ratio of the amount of material taken in from the medium to the amount of cells produced is larger than it is in a normal culture. Since the change in this ratio during inhibition is a summation of all changes in concentration of intermediates and increases of rate of production of all waste products, experimental measurement of it should be relatively easy.

The weights of cells per litre and the remaining concentrations of ammonium nitrogen (micro-diffusion method, Conway, 1961, accuracy ± 1 mg. N/litre) were measured at intervals in two sets of six unaerated cultures at pH 7·00 containing initially 95 mg. N/litre; the cultures of one set contained also 0·1 μM tetracycline. The results for all like media were in close correspondence so that composite weight of cells per litre against time, and concentration of ammonium nitrogen against time, graphs could be constructed. From these graphs corresponding values of weight of cells per litre and nitrogen consumed could be obtained. The results (Fig. 5) show that in fact, early in the growth phase in the presence of tetracycline a smaller proportion of the nitrogen taken in is utilised in the production of cells but that as the system stabilises the rate of intake of nitrogen decreases so that it more nearly matches its normal ratio to the rate of production of cells. In a similar experiment, aerated cultures containing oxytetracycline showed a similar deviation from the behaviour of the control cultures.

Discussion

Attributing the inhibitory power of the two tetracyclines solely to their molecular forms TH_2 and $\emptyset H_2$, accounts quantitatively for the effects of varying the concentration of magnesium and the pH of the medium. Thus the relative effectiveness of the two tetracyclines can only be examined by comparing the degrees of inhibition due to the same concentrations of TH_2 and $\emptyset H_2$. This comparison is made in Fig. 6.

Mode I tetracycline and mode I oxytetracycline are indistinguishable and since neither are affected by enrichment of the medium, nor by aeration, but both produce phenomena expected from a derangement of protein synthesis, it is probable that both tetracycline molecules equally inhibit the same reaction in the sequence producing protein from amino-acids. This being so, this particular function is probably associated with a part of the molecule which is the same in both tetracycline and oxytetracycline.

The two mode II inhibitors are quantitatively different; that for oxytetracycline has a lower threshold concentration and the degree of inhibition rises less steeply with concentration. However, both are eliminated by aeration and by the same enrichment of the medium and both affect the reduction of triphenyltetrazolium ions in the same way. Thus it is probably that both tetracycline molecules inhibit the same hydrogen-transfer reaction or the provision of a reducible substance. This being so, this particular function is probably associated with a part of the molecule which is affected by the difference of structure.

These considerations of molecular structure might well be expected if the primary step in inhibition is combination with enzymes; combination with one enzyme accounting for mode I and with another for mode II. The linearity or near linearity of portions of the Figs. 1, 2, 3 and 6 can be explained by the same hypothesis (Harris and Morrison, 1961).

Recent work with mice injected interperitoneally with tetracycline (Du Buy and Showacre, 1961), showed that the mitochondria of liver, spleen and brain cells became fluorescent and oxidative phosphorylation

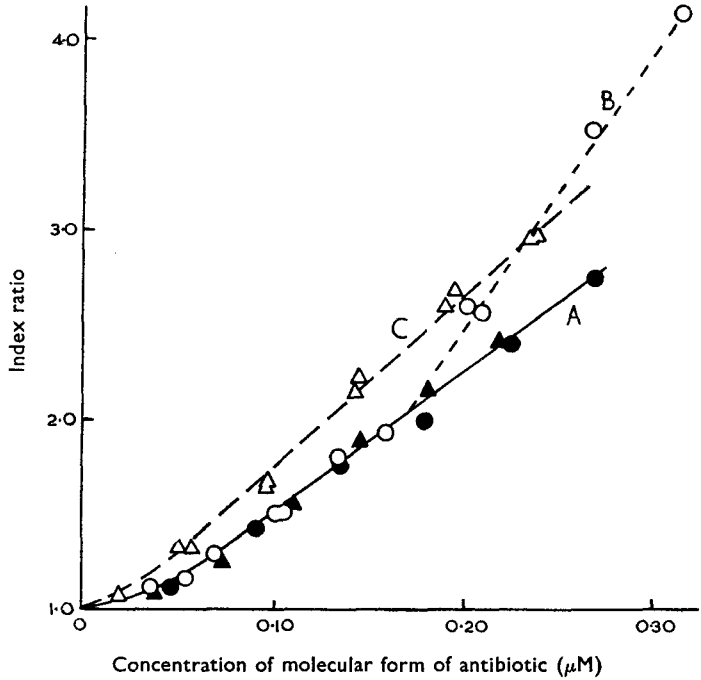


FIG. 6. Comparison of inhibitions of *A. aerogenes* by tetracycline and oxytetracycline. Cultures inhibited by tetracycline: ○, unaerated; ●, aerated. Cultures inhibited by oxytetracycline: △, unaerated; ▲, aerated. A, mode I, common to both antibiotics; B, mode II for tetracycline; C, mode II for oxytetracycline.

within them was decreased although oxygen uptakes were unaffected: the correspondence to the present mode II is obvious, and hydrogen-transferring enzymes or enzymes producing reducible substances might be expected to be present in the mitochondria.

In clinical cases any contributions of mode II to the cytotoxic action of these two tetracyclines can be only in tissues which do not have an adequate supply of oxygen, but mode I should contribute in all tissues that the antibiotics can reach. The power to combine with cations may be the cause of the absence of reports that these tetracyclines affect the faster growing cells of host animals such as blood cells which are affected by the inhibitor of protein synthesis, chloramphenicol: the tetracyclines may not be able

to reach the blood producing centres in the molecular inhibitory form because of the availability of calcium in the bone. Milch, Rall and Tobie (1957) report that interperitoneally injected tetracycline induces fluorescence in the bones of several species of laboratory animals, and we find that filtering an aqueous solution of tetracycline through powdered calcium ortho-phosphate severely decreases the inhibitory power.

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