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Action of 12 Tetracyclines on Susceptible and Resistant Strains of *Staphylococcus aureus*

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The relative growth inhibition caused by 12 tetracyclines in a susceptible strain of *Staphylococcus aureus* (111-elim) and in the same strain carrying a resistance-plasmid (111) showed entirely different patterns. For four of the tetracyclines (minocycline, anhydrotetracycline, chelocardin, and desdimethylaminotetracycline), the strain with the tetracycline plasmid (111) had virtually the same tolerance as the susceptible strain (111-elim). A resistant mutant of strain 111-elim showed a third pattern of relative growth inhibition, and another distinct pattern was observed in a veterinary wild strain of *S. aureus*. Of the 12 tetracyclines, 11 were effective inducers of higher tetracycline resistance in *S. aureus* 111, but no correlation was found between the efficacy of the tetracyclines as inducers and as inhibitors of growth of 111-elim or 111. At external drug concentrations causing doubling of the generation time (K_i), 111-elim accumulated tetracycline, oxytetracycline, and minocycline to a degree corresponding to several thousand molecules per coccus. At a fixed external drug concentration, 111 accumulated less tetracycline and oxytetracycline than 111-elim, whereas comparison at their respective K_i values showed accumulation to be significantly higher for 111 than for 111-elim. The accumulation of tetracyclines is assumed to involve both surface sorption and active membrane transport. Resistance is probably due to decreased accumulation of the drugs, and a hypothesis explaining the mechanism of resistance is offered.

Bacterial susceptibility to tetracyclines (Tcs) depends on the affinity of these drugs for at least two biological systems: the energy-dependent uptake mechanism (6, 9, 20) and the ribosomal protein-synthesizing machinery (13). Resistance may be obtained artificially by mutations influencing either of these systems (5, 15). In resistant wild strains of *Staphylococcus aureus*, two additional substances with affinity for Tc must be considered: the repressor of resistance (11, 12, 18) and the "resistance-antigen (1, 19).

It seems reasonable to assume that relative affinities of these four systems for various Tcs may vary independently. As a result, significant variations in relative susceptibility of different strains of *S. aureus* to a series of Tcs may be expected. Moreover, the pattern of inhibitory effect of these Tcs on a susceptible bacterial strain, and on the same strain transfected with a resistance factor, might be different.

These assumptions were examined in the present study in which the inhibitory activity of 12 Tcs on growth of susceptible and resistant strains and their influence on derepression of resistance were compared. For three of the Tcs, uptake was also investigated.

MATERIALS AND METHODS

Bacterial strains. The Tc-resistant *S. aureus* strain 111 and the susceptible *S. aureus* strain 111-elim, obtained by elimination of the Tc plasmid, have been described (1, 18, 20). From 111-elim, a resistant mutant was obtained by treatment of a suspension of bacteria during the exponential growth phase with 8 mM ethidium bromide for 60 min. After washing, a heavy inoculum of the bacteria was plated on nutrient agar (NA) with 0.5 μg of Tc/ml. A second-step mutation was obtained by transfer to NA + 5.0 μg of Tc/ml. The strain obtained, designated 111-mut, grew on NA + 10 μg/ml in 48 h at 37 C. The resistance of 111-mut could not be eliminated by growth in nutrient broth (NB) at elevated temperature or by addition of ethidium bromide. Six additional strains of *S. aureus* were also included in this study. Four of these strains were from human pathological lesions,

whereas two (V 738 and V 767) were isolated from bovine mastitis milk; all of the strains were identified as *S. aureus* according to microscopic and colonial morphology and positive coagulase reactions. The veterinary strains were producers of hot-cold beta-hemolysin that was potentiated by *Streptococcus agalactiae* (camp reaction).

Nutrient media and drugs. Difco NA and NB were used throughout. Relatively high concentrations of the Tcs were freshly prepared daily in distilled water (1,000 μg/ml), methanol (doxycycline at 19.5 mg/ml, methacycline at 9.5 mg/ml, and chelocardin at 2.0 mg/ml), or dioxane (desdimethylamino-Tc at 50 mg/ml). These solutions were diluted with phosphate buffer (NaCl, 8.0 g; K_2HPO_4, 1.21 g; KH_2PO_4, 0.34 g; in 1,000 ml of water; pH 7.3). In appropriate control examinations, it was shown that the minute amounts of dioxane and methanol transferred to NB with the solutions of some of the Tcs had no influence on the growth rate of 111-elim and 111.

Most of the Tcs studied were kindly provided by Lederle Laboratories (demethyl-Tc, demethylchlor-Tc, Tc, chlor-Tc, and minocycline) and Pfizer Co., Inc. (oxy-Tc, doxycycline, and methacycline) through their local agencies (Neopharm Ltd., and Nissan Preminger Ltd., respectively). Samples of three of the Tcs not in clinical use (desdimethylamino-Tc, 5a, 6-anhydro-Tc, and 4-epi-Tc) were obtained through the generosity of V. A. Haverbeke, Pfizer, Brussels, Belgium, and a sample of chelocardin (2-decarboxamido, 2-acetyl, 4-desdimethylamino, 4-epiamino, 9-methyl, 5a,6-anhydro-Tc) was obtained through the courtesy of A. D. Geizler, Scientific Division, Abbott Laboratories, North Chicago, Ill. For convenience, the structures of the Tcs used are shown in Fig. 1. Tc-7-^{3}H(N) was purchased from the Radiochemical Centre, Amersham, Bucks., England; this radioproduct is delivered as a powder. Tc-^{3}H(G), oxy-Tc-^{3}H(G), and minocycline-^{3}H(G) were produced in Israel (Uri Buchman, Nuclear Research Center, Negev) and delivered as a methanol solution. These products were dried under vacuum at 37 C and stored in small portions in sealed ampoules until use. Radioactivity of these tritiated Tcs chromatographed purely (≥98%) in two solvent systems (*n*-butanol-ethanol-water, 10:10:5, and *n*-butanol saturated with water). R_f values were identical to those of the corresponding nonradioactive substances. E_{380} and inhibition of bacterial growth by ^{3}H-Tcs(G) proved likewise quantitatively equivalent to findings obtained with nonradioactive compounds on a weight to weight basis. Specific activities were 590 and 1,400 mCi/mmol for ^{3}H-Tc-7 (two batches), 47.90 mCi/mmol for ^{3}H-Tc(G), 13.92 mCi/mmol for ^{3}H-oxy-Tc(G), and 10.78 mCi/mmol for ^{3}H-minocycline(G). The radioactive Tcs were stored dry, and small quantities were dissolved in water before each experiment. Quantitation was performed according to radioactivity.

Accumulation of Tcs. Bacterial accumulation of Tcs was examined with early log-phase cultures in NB. In some experiments, 100 μg of chloramphenicol/ml was first added, incubation was continued for 15 min, and thereafter the tritiated Tcs were introduced; in this way, equal inhibition of susceptible and resistant cultures was ensured. The bacteria were incubated with the antibiotic agents for 15 min at 37 C (mechanical aeration). In other experiments, we were interested in studying uptake of Tcs during growth. In these cases, the bacteria were

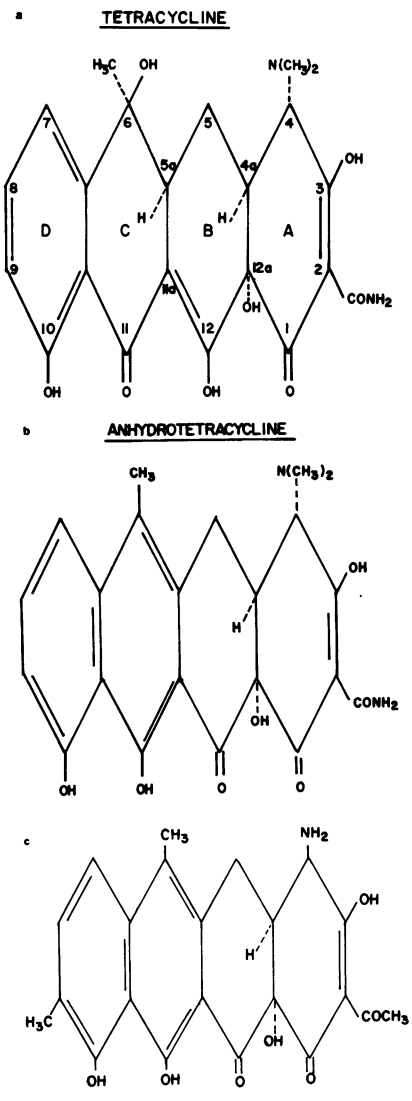

FIG. 1. *Chemical structure of 12 tetracyclines used in this study. (a) Tetracycline (Tc). Chlor-Tc is 7-Cl. Oxy-Tc is 5-oxy. The OH groups of 5 and 6 are in trans position. Doxycycline: 6-deoxy, 5-oxy-Tc. Minocycline: 6-deoxy, 6-demethyl, 7-dimethylamino-Tc. Demethyl-Tc and demethylchlor-Tc are 6-demethyl compounds. Methacycline: 6-demethyl, 6-deoxy, 6-methylene, 5-oxy-Tc. (b) Anhydro-Tc. (c) Chelocardin.*

grown overnight in NB with the required drug concentration. Subsequently, they were grown in drug-containing NB until they reached the exponential growth phase, diluted in prewarmed NB + ^{3}H-Tcs to an initial optical density of 0.02 (660 nm), and incubated for 90 min as described for induction of high-level resistance.

After incubation, the bacteria were cooled rapidly in an ice bath, sedimented by centrifugation at 4 C (15,000 × *g* for 15 min), washed in one portion of 10 ml of cold phosphate buffer, and finally resuspended in 3 ml of this buffer. Turbidity was determined with a Klett-Summerson spectrophotometer (540 nm), *n*-butanol was added to a final concentration of 5% (7), and after 15 min two 1-ml portions were taken for determination of radioactivity in a Tri-Carb scintillation spectrophotometer, model 3950. Instagel emulsifier (15 ml; Packard Instrument Co., Inc., Downers Grove, Ill.) was added to each of the scintillation vials.

Quantitation of antibiotic activity. The growth rate coefficient k_0 in NB (mechanical aeration, 37 C) was determined as usual:

$$k_0 = \frac{2.303}{t} \times (\log E - \log E_0)$$

E_0 and E refer to optical densities at 660 nm at the time of addition of the drug and t min later, respectively (in this study, t = 90 min). All determinations were performed on cultures in the exponential growth phase at low turbidity (initial optical density, 0.015 to 0.020) obtained by dilution of a mid-log culture in prewarmed NB. From the growth curves in plain NB and in NB with graded drug concentrations, the correlation between drug concentration and apparent growth rate coefficient, k, was determined, by use of four to six simultaneous growth curves in NB with different drug concentrations. The k values were recorded as percentages of k_0. When k was plotted against the log of drug concentration, a sigmoid curve was obtained; to determine its exact course, a great number of points had to be determined experimentally. Therefore, the k values were transformed to probits (8). In accordance with the observations of Garrett, Miller, and Brown (10), fair proportionality was obtained between the probit of k and the log of drug concentration. The growth rate coefficient (k) at a certain drug concentration could therefore be predicted by interpolation even if the growth inhibition was determined only at two different concentrations. Use of a greater number of experimental observations allowed more precise determination of the position of the line.

The antibiotic activity of a drug can be indicated by the concentration (K_i) which causes a doubling of the generation time (k = 50% or probit of k = 5.0). Since the molecular weights of all drugs examined are rather similar, K_i in this paper is indicated as micrograms per milliliter.

Induction of increased resistance. Resistant *S. aureus* cultures can be induced to increased levels of resistance by contact with the drug in subinhibitory concentrations. When high-level resistance is desired, the induction period is prolonged, since drug added at the required relatively high concentration inhibits protein synthesis and, therefore, development of resistance (18). In the present study, induction with relatively low drug concentrations ($\leq$1 μg/ml) was achieved in the following way. Exponential NB cultures were diluted to a turbidity of 0.02 optical density (660 nm) in prewarmed drug-containing NB and incubated for 90 min at 37 C (mechanical aeration). The bacteria were thereafter sedimented by centrifugation and resuspended in prewarmed portions of NB with or without the drug for determination of resistance as described above. Preliminary examinations had shown that, for concentrations below the K_i of the culture, growth in Tc-containing NB did not induce the culture to a higher resistance level during 24 h than did growth at the same drug concentration during 90 min. For induction with higher concentrations than the K_i, the cultures were grown overnight on NA with the desired drug concentration; the cocci were then suspended in fresh drug-containing NB and grown to the logarithmic growth phase (90 min). The bacteria were thereafter sedimented and resuspended in NB with and without the drug for determination of resistance.

Since the highly resistant cultures obtained in this way returned to the original resistance level after a few generations in drug-free NB (18), the increase in resistance obtained was probably due to derepression of the Tc-resistance gene and not to selection of the most resistant individuals of a heterogeneous population.

RESULTS

Susceptibility of S. aureus to various tetracyclines. Plots of k versus log of concentrations of various Tcs are shown in Fig. 2 for *S. aureus* 111-elim, 111-mut, and 111. Table 1 contains K_i values for various Tcs, both for the strains mentioned and for two strains isolated from bovine mastitis milk (V767 and V738). The figures in the table represent in most cases mean values of several independent determinations; when they were based on more than five experiments, standard deviations of the mean are also given.

Greatest inhibition of 111-elim was obtained with doxycycline, methacycline, chlor-Tc and demethylchlor-Tc, with K_i of 0.032 to 0.036 μg/ml. K_i for Tc was 0.080. Oxy-Tc and demethyl-Tc were slightly less active than Tc, and anhydro-Tc, desdimethylamino-Tc, chelocardin, and epi-Tc were 7 to 15 times less active than Tc, with a K_i of 1.22 μg/ml for epi-Tc. It should be particularly emphasized that for 111-elim the K_i for minocycline was 67% of the value for Tc.

For the resistant *S. aureus* 111, K_i values varied much more: from 0.071 μg/ml for minocycline, to 15.0 μg/ml for epi-Tc. The susceptibil-

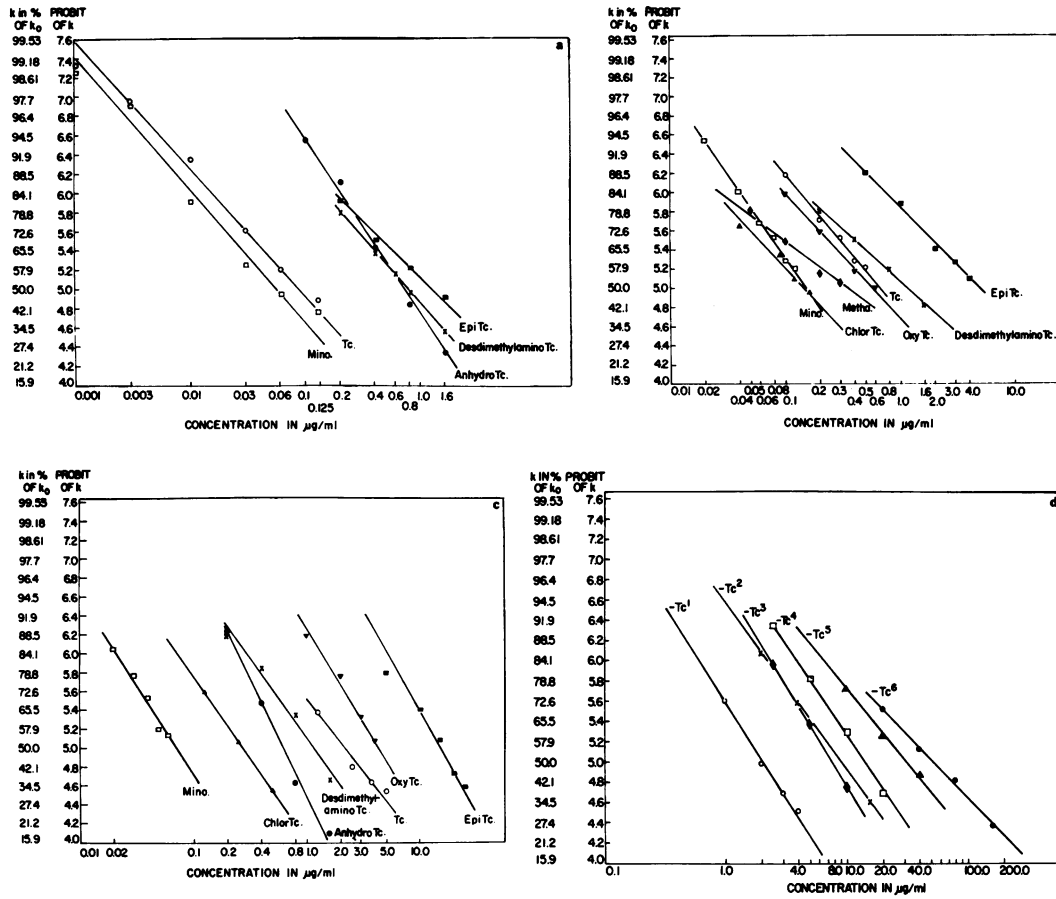

FIG. 2. *Plots of the apparent growth rate coefficient k against the concentration of the drug in the medium. The scale is probit-log. (a) Staphylococcus aureus 111-elim. (b) S. aureus 111-mut. (c) S. aureus 111. (d) S. aureus 111. Resistance to tetracycline (Tc) after induction. Tc¹ = no induction. Tc² = induction with 0.03 µg of desdimethylamino-Tc/ml. Tc³ = induction with 2.0 µg of epi-Tc/ml. Tc⁴ = induction with 0.003 µg of minocycline/ml. Tc⁵ = induction with 10 µg of oxy-Tc/ml. Tc⁶ = induction with 10 µg of Tc/ml.*

TABLE 1. *Susceptibility of Staphylococcus aureus strains to 12 tetracyclines (Tcs)*[a]

Strain	Doxy-cycline	Chlor-Tc	De-meth-yl-chlor-Tc	Metha-cycline	Mino-cycline	Tc	De-meth-yl-Tc	Oxy-Tc	Chelo-cardin	Anhy-dro-Tc	Des-di-methyl-amino-Tc	Epi-Tc
111-elim	0.032	0.035	0.036	0.036	0.0529 ± 0.0043[b]	0.080 ± 0.011[b]	0.125	0.138	0.57	0.70	0.75	1.22
111	0.30	0.275	0.87	0.80	0.0713 ± 0.009[b]	2.260 ± 0.154[b]	3.50	4.30	0.65	0.63	1.15	15.0
	(9.4)	(7.9)	(24)	(22)	(1.4)	(28)	(28)	(31)	(1.1)	(0.89)	(1.5)	(12)
111-mut	0.54	0.14	0.52	0.42	0.162	0.66	1.06	0.85	—	0.42	1.15	5.00
	(17)	(4.0)	(14)	(12)	(3.1)	(8.3)	(8.5)	(6.2)		(0.60)	(1.5)	(4.1)
V 738	0.78	0.88	0.42	0.50	0.210	4.35	1.24	1.70	—	0.64	2.2	15.0
	(24)	(25)	(12)	(14)	(4.0)	(54)	(9.9)	(12)		(0.91)	(2.9)	(12)
V 767	0.14	0.21	0.67	0.65	0.056	1.8	0.92	3.50	—	0.48	—	—
	(4.4)	(6.0)	(19)	(18)	(1.1)	(23)	(7.4)	(25)		(0.69)	—	—

[a] The figures indicated K_i values, i.e., concentrations (µg/ml) of drug causing a doubling of generation time. Ratios between K_i of resistant strains and K_i of the susceptible strain (111-elim) are indicated in parentheses.

[b] K_i ± standard deviation.

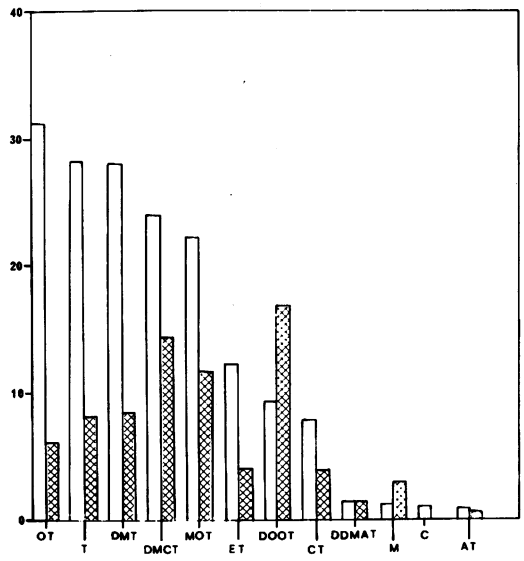

FIG. 3. *Resistance pattern of Staphylococcus aureus 111 and S. aureus 111-mut to 12 tetracyclines. The figures indicate the fraction K_i 111/K_i 111-elim (open bars) or K_i 111-mut/K_i 111-elim (shaded bars). OT = oxytetracycline; T = tetracycline; DMT = demethyltetracycline; DMCT = demethylchlortetracycline; MOT = methacycline; ET = 4-epitetracycline; DOOT = doxycycline; CT = chlortetracycline; DDMAT = desdimethylaminotetracycline; M = minocycline; C = chelocardin; AT = anhydrotetracycline.*

ity of 111-elim and 111 to minocycline, anhydro-Tc, chelocardin, and desdimethylamino-Tc varied only slightly. The resistance mechanism of 111 seems therefore to be inefficient against these compounds. For the other drugs, the K_i of 111 was 8 to 31 times greater than K_i of 111-elim (see Table 1). A third pattern of susceptibility to various Tcs was observed in *S. aureus* 111-mut (Table 1, Fig. 3). For Tc, the drug used in the selection of this resistant mutant, the K_i was 3.5 times lower than in 111, but for minocycline it was 2.5 times higher. All in all, the variations in K_i values were relatively slight for 111-mut, from 0.162 μg/ml (minocycline) to 1.15 μg/ml (desdimethylamino-Tc), with the exception of epi-Tc (K_i = 5.0 μg/ml). The resistance pattern of V767 was comparable to that of 111, but V738 showed a clearly distinct pattern. K_i-V738/K_i-V767 was 0.63 to 0.64 for methacycline and demethylchlor-Tc and was 1.5 for Tc, but was 3.5 to 4.0 for minocycline, chlor-Tc and doxycycline. Thus, the five strains of Table 1 represent four distinct patterns of relative susceptibility to Tcs.

For other resistant strains examined, only the K_i for Tc and minocycline was determined (Table 2). K_i values for Tc varied between 1.2 and 39.0 μg/ml, and for minocycline between 0.03 and 0.32 μg/ml. In all of these strains, resistance could be significantly increased by previous growth of the cells in NB + Tc. In none of the strains in Table 2 was the fraction K_i of Tc/K_i of minocycline as low as in V738, though this value varied widely among the strains.

Induction of increased Tc resistance. By growth in drug-containing NB, all resistant wild strains examined could be induced to exhibit increased levels of resistance (Tables 2 and 3). This was not the case with 111-mut. The relationship between concentrations of various Tcs in NB during induction and the resistance level obtained is shown in Fig. 4. Over a certain gradient of concentrations, linearity was observed between log of drug concentrations and apparent K_i values. By extrapolation, the lowest concentration causing an increased resistance can be determined. This value was calculated as 0.16 ng/ml for minocycline. Experimentally, 0.3 ng/ml had a definite inducing effect, whereas the effect of 0.1 ng/ml was almost negligible. It should be noted that the slope of Tc induction by minocycline was steeper than that of induction by Tc; this may be due to the steeper slope of bacterial minocycline accumulation than of Tc accumulation (see Fig. 5 and 7). Induction of minocycline resistance by Tc had a particularly slight slope. This probably indicates that the resistance material synthesized has lesser activity for minocycline than for Tc; this interpretation is consistent with the fact that K_i of 111 for minocycline was only slightly higher than that of 111-elim. The highest levels of Tc resistance obtained by induction correspond to K_i values of approximately 50 μg/ml. This value is a

TABLE 2. *Inhibition of Staphylococcus aureus strains by tetracycline and minocycline*[a]

Strain	Induction	K_i		K_i, tetracycline/K_i minocycline
		Tetracycline	Minocycline	
92	–	6.0	0.16	37
102	–	1.2	0.030	40
	+	—	0.29	
103	–	30.0	0.10	300
	+	—	0.32	
108	–	39.0	0.32	122
	+	108.0	1.0	108

[a] Figures represent K_i values, i.e., drug concentrations (μg/ml) causing doubling of generation time in nutrient broth without previous induction, or after previous induction with 10 μg of tetracycline/ml.

TABLE 3. *Inhibition of Staphylococcus aureus 111 by tetracycline after induction with various tetracyclines*

Inducer	Concn of inducer (μg/ml)	Apparent K_i values, tetracycline
No tetracycline	—	2.248 ± 0.148[a]
Tetracycline	0.003	4.0
	0.01	5.8
	0.03	6.7
	0.1	9.3
	10.0	52.0
Doxycycline	0.003	7.1
Chlortetracycline	0.001	5.1
Methacycline	0.001	4.4
Demethylchlortetracycline	0.003	5.7
Minocycline	0.0001	3.1
	0.0003	3.9
	0.001	8.8
	0.003	11.7
Oxytetracycline	0.003	2.0
	0.03	4.6
	1.0	17.0
Demethyltetracycline	0.003	4.6
Chelocardin	0.1	2.6
	0.3	2.8
Anhydrotetracycline	0.001	2.7
	0.03	9.5
Desdimethylaminotetracycline	0.03	7.2
Epitetracycline	2.0	7.6

[a] K_i ± standard deviation.

600-fold increase above the level of 111-elim and 20-fold above the basic level of 111. Figure 4 does not indicate that this value is the maximum obtainable, but higher concentrations of the drugs used for induction were too inhibitory for the conducting of meaningful experiments.

In Table 3 are compiled apparent K_i values after induction with various Tcs included in this study. The variations in efficacy as inducers were obvious. Minocycline was the best inducer of all. Oxy-Tc was less effective than anhydro-Tc and desdimethylamino-Tc. 4-Epi-Tc proved to be a particularly weak inducer, and with chelocardin no significant induction was obtained with a drug concentration corresponding to one-half the K_i of 111.

It should be emphasized that these results give no information about the relative affinity of various Tcs to the repressor of resistance; for this purpose, we need to know both the concentration of repressor-bound drug and the intracellular concentration of free drug with which it is in equilibrium. It is, however, not practical to determine the accumulation of Tcs by the bacteria after incubation with low drug concentrations such as those used for induction, unless radioactive compounds are available. Therefore, only uptake of Tc, oxy-Tc, and minocycline was studied.

Accumulation of Tcs. Uptake of tritiated Tcs by 111-elim and 111 was, in some of the

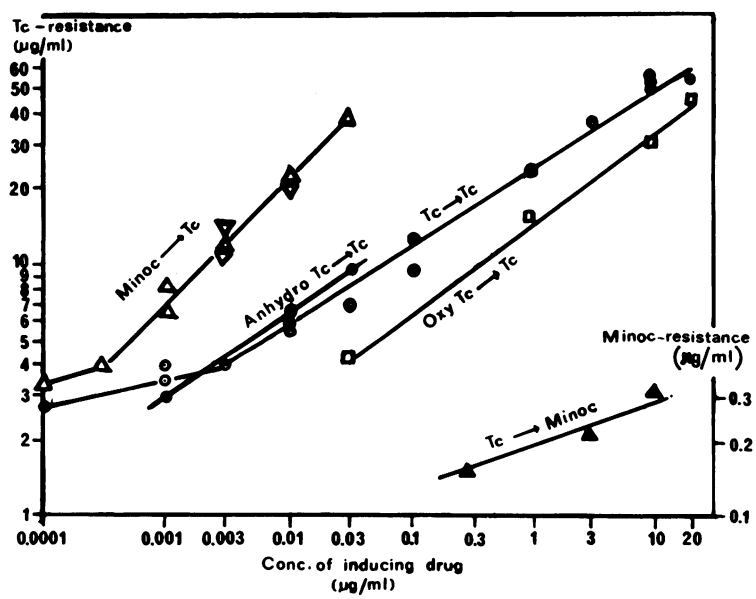

FIG. 4. *Induction of Staphylococcus aureus 111 to increased resistance to tetracycline (Tc) or minocycline. (△) Induction of Tc resistance by minocycline. (⊙) Induction of Tc resistance by Tc. (□) Induction of Tc resistance by oxy-Tc. (●) Induction of Tc resistance by anhydro-Tc. (▲) Induction of minocycline resistance by Tc. The abscissa shows concentrations of the inducers, the ordinate, and the K_i values obtained.*

experiments, examined with bacteria in the exponential growth phase charged with various concentrations of the Tcs plus 100 μg of chloramphenicol/ml. In other experiments, the accumulation of ^{3}H-Tc and ^{3}H-oxy-Tc by 111 was studied in exponentially growing cultures in which resistance to the concentration of the drug added had been induced. Over the whole range of concentrations examined (0.1 to 10 μg/ml), the induced cultures accumulated about 12% of the Tc accumulated by the susceptible 111-elim (Fig. 5). At low external drug concentrations, the accumulation of the resistant 111 (the chloramphenicol-inhibited culture) was only slightly greater than that of the growing induced culture, but at relatively high external concentrations it came closer to that of 111-elim. If 1 mg (dry weight) corresponds to 4 μliters of cytoplasm, then at an external concentration of 1 μg/ml the drug was concentrated about 30 times by 111-elim, 4 times by the induced 111, and 10 times by the chloramphenicol-inhibited 111.

Binding of Tc by 111-elim was biphasic; at concentrations up to 10 μg/ml, the slope of binding against concentration was 0.8, but at higher concentrations it was about 2.3. A similar biphasic binding of Tc and oxy-Tc by *Escherichia coli* has been described by De-Zeeuw (6). No tendency to saturation of uptake was observed, although at the highest external concentrations that could be kept in stable solution excessive amounts of the drug, such as 130 and 250 μg/mg (dry weight), were accounted for by 111 and 111-elim, respectively. It is scarcely probable that these amounts of Tc are soluble in the protoplasm.

It should be noted that ^{3}H-Tc(G) and ^{3}H-Tc(7) were accumulated to about the same degree; experiments with the ^{3}H-Tc(G) compound gave in most instances slightly higher results than with ^{3}H-Tc(7). Several hypothetical forms of metabolism might have caused a significant difference between the calculated uptake of ^{3}H-Tc(G) and ^{3}H-Tc(7), but, of course, a negative result is insufficient to disprove metabolic transformation of the drug.

At external concentrations below 10 μg/ml, the accumulation of oxy-Tc by 111-elim was only about 50% of that of Tc (Fig. 6). The uptake curve for oxy-Tc was also biphasic. For all concentrations examined, the induced grow-

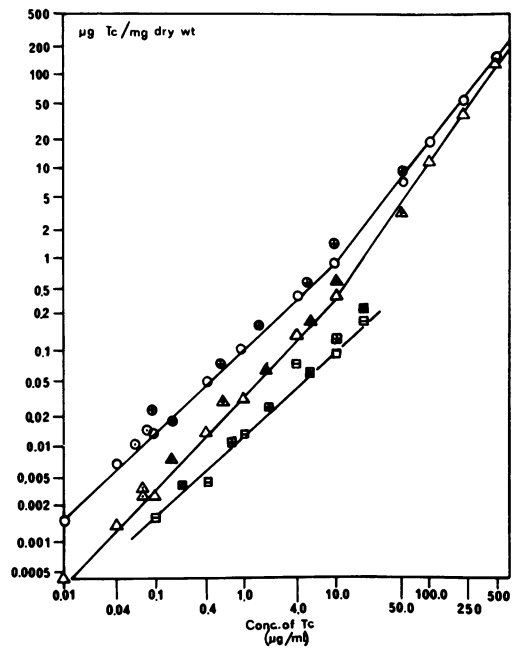

FIG. 5. *Accumulation of tetracycline (Tc) by the susceptible Staphylococcus aureus 111-elim (O) and the resistant S. aureus 111 (Δ) at different external drug concentrations; to these cultures were added 100 μg of chloramphenicol/ml. The squares indicate accumulation by S. aureus 111, induced to high-level resistance, during exponential growth in the presence of the indicated drug concentrations. The empty symbols indicate experiments with ^{3}H-Tc(7), and the pointed symbols experiments with ^{3}H-Tc(G).*

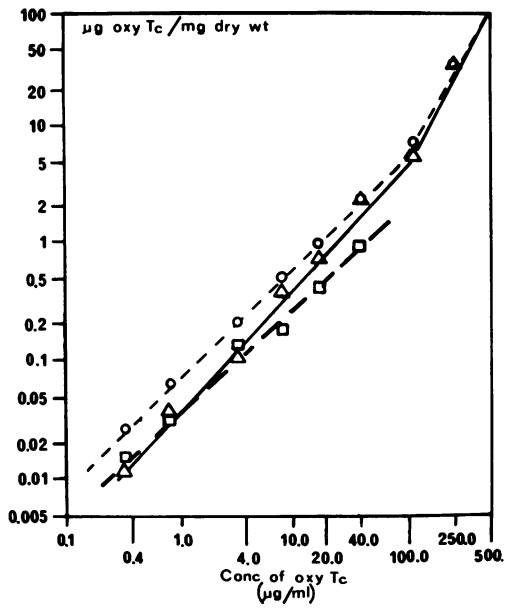

FIG. 6. *Accumulation of oxytetracycline (oxy Tc) by the susceptible Staphylococcus aureus 111-elim (O) and the resistant S. aureus 111 (Δ), both inhibited with 100 μg of chloramphenicol/ml. The squares indicate accumulation by S. aureus 111, induced to high-level resistance, during exponential growth in the presence of the indicated drug concentrations.*

ing culture of 111 accumulated about 50% as much of this drug as the susceptible 111-elim. For 111 inhibited with chloramphenicol, the results were similar to those of the induced culture at low external concentrations, but they approached those of the susceptible one at high concentrations.

Figure 7 shows the dependence between the external concentrations of ^{3}H-minocycline and accumulation of this drug in 111-elim and 111, both inhibited by chloramphenicol. Because of the low specific activity of the tritiated minocycline, we were unable to test the accumulation at extremely low drug concentrations which enabled growth of 111. At 0.1 μg/ml, 111-elim accumulated 10 ng of minocycline per mg (dry weight), which is less than the uptake of Tc. However, the slope of uptake was 1.5, and at a concentration of 50 μg/ml accumulation of minocycline was several times greater than of Tc. With minocycline, saturation of uptake was observed at an external concentration of about 200 μg/ml; the accumulation at this concentration corresponded to 120 μg per mg (dry weight) of 111-elim. At drug concentrations up to 50 μg/ml, the uptakes by 111-elim and 111 were not significantly different, but at saturation 111 contained significantly less minocycline than did 111-elim.

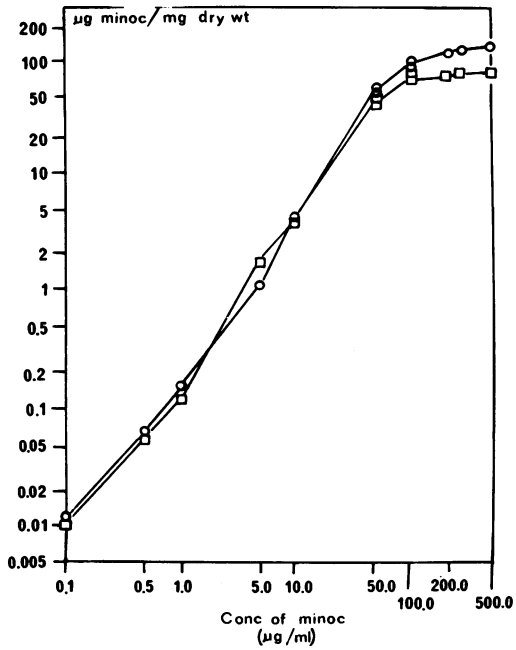

FIG. 7. *Accumulation of minocycline by the Tc-susceptible Staphylococcus aureus 111-elim* (O) *and the Tc-resistant S. aureus 111* (□), *both inhibited with 100 μg of chloramphenicol/ml.*

DISCUSSION

The Tcs examined in this study can be divided into two groups, one consisting of those drugs which are used in medical practice and the other of those that are not (anhydro-Tc, chelocardin, 4-epi-Tc, and desdimethylamino-Tc). The concentratiion (K_i) causing 50% inhibition of the growth rate of the susceptible *S. aureus* 111-elim varied between 0.032 and 0.138 μg/ml in the first group, and between 0.57 and 1.22 μg/ml in the other. The chloratom at C_7 of chlor-Tc seemed to have a marked effect, decreasing the K_i to less than 50%; three Tcs without oxygen at C_6 had similar low K_i values (minocycline, doxycycline, and methacycline). Demethylation of C_6 seemed to cause a slight increase of K_i for 111-elim. Aromatization of the C-ring (chelocardin, 5a,6-anhydro-Tc) strikingly decreased efficacy. The effect of the C_4-dimethylamino group was illustrated by the high K_i values observed for desdimethylamino-Tc and 4-epi-Tc. With a cell-free extract of *E. coli* B, Rifino et al. (16) showed that 4-epi-Tc and desdimethylamino-Tc are less effective than Tc in inhibiting messenger ribonucleic acid-directed phenylalanine incorporation.

The relative efficacy of these drugs for the resistant 111 showed an entirely different pattern, a fact indicating that resistance cannot simply be due to inhibition of the Tc-transport system of 111-elim. Most impressive was the fact that the K_i of 111 for chelocardin, anhydro-Tc, minocycline, and desdimethylamino-Tc was only 0.9 to 1.5 of that of 111-elim (Table 1); these compounds inhibited 111 much better than Tc. In a comprehensive review of structure-activity relationships in Tcs, Blackwood and English (2) noted the low minimal inhibitory concentrations of minocycline, 5a,6-anhydro-Tc, and desdimethylamino-Tc for Tc-resistant strains of *S. aureus*; they correlated this with the lipophilic nature of these compounds. Resistance to Tc may be due either to the action of a basic amount of a "resistance-substance" (1) or to the induction of resistance *during* determination of the resistance level. Accordingly, relative susceptibility of a Tc-resistant strain to a certain Tc analogue might reflect either lack of activity of the resistance mechanism for this compound or lack of efficacy of the compound as an inducer. The data compiled in this study leave the latter possibility open only

for chelocardin, since minocycline, desdimethylamino-Tc, and 5a,6-anhydro-Tc were effective inducers of Tc resistance in 111 (Table 3).

Chlor-Tc, doxycycline, and epi-Tc composed a group with an intermediate relationship between the K_i of 111-elim and 111 (K_i of 111:K_i of 111-elim = 8 to 12). Interestingly, this ratio was 24 for demethylchlor-Tc. This drug also had significantly less activity than chlor-Tc for 111-mut and V767, but more activity for V738 (Table 1). Demethyl-Tc caused significantly greater inhibition than Tc of both veterinary strains (V767 and V738). Strain 111 was less resistant to Tc, minocycline, chlor-Tc, doxycycline, and desdimethylamino-Tc than V738, but significantly more so to oxy-Tc, methacycline, demethyl-Tc, and demethylchlor-Tc. Either the basic principle of the resistance mechanism is not identical in all resistant wild strains or clinical use of various Tc analogues has led to selection of strains with minor molecular variations in the structure of the "resistance-substance."

A comparison between Tables 1 and 3 reveals no correlation between inhibition of 111-elim or 111 and efficacy as inducers. A few examples will illustrate this. Minocycline with a lower K_i for 111-elim than Tc was markedly more inductive, but anhydro-Tc with a significantly higher K_i for 111-elim was no less inductive than Tc. The resistance mechanism was inactive with both minocycline and desdimethylamino-Tc, but the former was the best inducer among all drugs studied whereas the latter was a very weak inducer. Minocycline, anhydro-Tc, and desdimethylamino-Tc must be considered as gratuitous (or almost gratuitous) inducers of Tc resistance, since they do not interact with the "resistance-substance."

It has been questioned whether compounds with an aromatic C ring belong to the Tcs (2). The problem is meaningless in such general terms; it must be specified in relation to each of the biological systems with which Tc interacts. It should be considered whether these compounds compete with Tc for uptake (a task not so simple to undertake since uptake is not saturating) and for binding to ribosomes (3); it should also be examined whether the Tc-resistant strain has increased tolerance to these compounds and whether they act as specific derepressors of Tc resistance. Chelocardin clearly lacks the two latter properties of Tcs. On the other hand, 5a,6-anhydro-Tc and all other compounds studied are true Tcs, at least as far as their action as specific inducers of Tc-resistance is concerned.

From Fig. 5–7, it can be calculated (in part by extrapolation) that 111-elim accumulated 14 ng of Tc/mg (dry weight), 9 ng of oxy-Tc/mg (dry weight), and 6 ng of minocycline/mg (dry weight) when the drug concentrations corresponded to the respective K_i values. If 1 mg (dry weight) represents 3×10^9 cocci, then these figures correspond to about 6.1×10^3, 4.0×10^3, and 2.6×10^3 molecules of drug per coccus. Accumulation of Tc and oxy-Tc by 111 was approximately 110 ng/mg (dry weight) and 160 ng/mg (dry weight) at the respective K_i values for these drugs. Moreover, after induction to higher resistance, 111 grew exponentially even under conditions leading to accumulation of 180 ng/mg (dry weight) of Tc and of more than 1,800 ng/mg (dry weight) of oxy-Tc (Fig. 5 and 6). The fact that a resistant culture is able to grow in spite of accumulation of many times the quantity of the drug sufficient to inhibit a susceptible strain has been emphasized (1, 17). This apparently contradicts the concept that the resistance mechanism functions by decreasing the drug permeation.

DeZeeuw (6), noting that accumulation of Tc and oxy-Tc by *E. coli* was a biphasic function of drug concentrations in the medium, assumed that binding at external concentrations less than the minimal inhibitory concentration was due to physical adsorption to the cell surface, but at higher concentrations it was due to energy-dependent concentrative uptake. In resistant strains, this active uptake was thus initiated at higher external concentrations than in susceptible ones. Such interpretations cannot explain our results with *S. aureus*, although accumulation of Tc and oxy-Tc by 111-elim was also biphasic. An external concentration corresponding to the K_i was well within the zone of the first phase, and only a few thousands of drug molecules per coccus were bound at the K_i. To explain the doubling of generation time, we must assume that all, or almost all, molecules were bound to susceptible sites of vital importance for the cell, probably to ribosomes. Moreover, induction of 111 to higher resistance was obtained by incubation of the cultures with drug concentrations leading to the binding of only a few hundred or less molecules per coccus. It is not likely that Tcs could cause derepression without penetrating the cell surface.

On the other hand, it is still most likely that Tc resistance is in some way due to decreased drug permeation. All attempts to demonstrate drug metabolism have been negative (1, 3, 6, 19) and ribosomes of susceptible and resistant bacteria bind the drug equally well (4, 14). Cell-

free systems for protein synthesis prepared with ribosomes from susceptible and resistant wild strains have also been reported to be equally sensitive to Tc (9, 12, 13).

To explain our results, we offer an explanation at variance with that proposed by DeZeeuw (6). The drug accumulation observed with exponentially growing induced cultures of 111 (Fig. 5 and 6) must essentially be due to binding of the drug to the cell surface while the ribosomes remained inaccessible. Whether this is due to an increased transport barrier or to stimulation of transport inside out cannot be stated on the basis of current information. At any rate, surface sorption seems to be a linear function of drug concentration in the culture medium. We assume that in susceptible cultures adsorption reaches the same degree as in resistant ones; the drug accumulation in 111-elim is thus the sum of drug bound to the surface and amount transported to the cytoplasm. The active transmembranal transport is probably a slower process than physical surface sorption and therefore a constant function of the drug reservoir on the cell surface (Fig. 5 and 6). Accumulation of Tc by the resistant, uninduced culture, incubated with chloramphenicol was, at relatively low drug concentrations, quantitatively similar to that of the induced culture, probably because of a basic resistance level, but at higher external concentrations this culture was more similar to 111-elim. The much steeper slope of accumulation observed at high drug concentrations, should be interpreted with due caution, since it corresponded to extremely high cellular concentrations. It seems more than questionable whether the drug is soluble in the cytoplasm at such levels. It seems not impossible that we are dealing with precipitation of the drug. Gram-stained preparations of *S. aureus* 111-elim, incubated 15 min with 500 μg of Tc/ml at 37 C, showed a strikingly polymorph picture with a high proportion of swollen or deformed cocci. This was not the case when the culture was incubated at 0 C with the same drug concentration. Further data, not reported in this communication, showed that Tc accumulation at this external drug concentration was markedly lower at 0 C than at 37 C. It should also be recalled that, even at the highest concentrations examined, the resistant culture accumulated significantly less Tc than the susceptible 111-elim, a fact which probably indicates that precipitation—if that is what happens—occurs intracellularly after transmembranal transport. It is therefore understandable that DeZeeuw (6) could abolish the second phase of accumulation with sodium azide and by heating the bacteria. At least with staphylococci, this treatment has also a significant influence on the first phase (to be published).

With minocycline, biphasic accumulation was not observed, and saturation was obtained when the external concentration reached 100 to 200 μg/ml. The intracellular accumulation reached 70 to 120 μg/mg (dry weight). At low external concentrations (0.1 to 1.0 μg/ml), 111 accumulated amounts of minocycline comparable to those of Tc; the high efficacy of minocycline as an inducer of Tc resistance was therefore probably due to a true affinity to the repressor. For external concentrations up to 50 μg/ml, no significant difference in accumulation of minocycline was obtained between 111 and 111-elim. This should, of course, be expected if resistance really is related to the degree of accumulation, since the K_i for 111 was only slightly higher than for 111-elim. At saturation, 111 contained markedly less minocycline than 111-elim; we have no plausible explanation for this.

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