

Molecular Basis of Tetracycline Action: Identification of Analogs Whose Primary Target Is Not the Bacterial Ribosome

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Tetracycline analogs fell into two classes on the basis of their mode of action. Tetracycline, chlortetracycline, minocycline, doxycycline, and 6-demethyl-6-deoxytetracycline inhibited cell-free translation directed by either *Escherichia coli* or *Bacillus subtilis* extracts. A second class of analogs tested, including chelocardin, anhydrotetracycline, 6-thiatetracycline, anhydrochlortetracycline, and 4-epi-anhydrochlortetracycline, failed to inhibit protein synthesis *in vitro* or were very poor inhibitors. Tetracyclines of the second class, however, rapidly inhibited the *in vivo* incorporation of precursors into DNA and RNA as well as protein. The class 2 compounds therefore have a mode of action that is entirely distinct from the class 1 compounds, such as tetracycline that are used clinically. Although tetracyclines of the second class entered the cytoplasm, the ability of these analogs to inhibit macromolecular synthesis suggests that the cytoplasmic membrane is their primary site of action. The interaction of class 1 and class 2 tetracyclines with ribosomes was studied by examining their effects on the chemical reactivity of bases in 16S rRNA to dimethyl sulfate. Class 1 analogs affected the reactivity of bases to dimethyl sulfate. The response with class 2 tetracyclines varied, with some analogs affecting reactivity and others (chelocardin and 4-epi-anhydrotetracycline) not.

The tetracyclines are a group of broad-spectrum antibiotics which are generally considered to prevent bacterial growth by inhibiting protein synthesis. This results from binding of antibiotic to a single site in the 30S ribosomal subunit which prevents attachment of aminoacyl tRNA to the ribosomal acceptor site (3). In order to reach the ribosome, these antibiotics must traverse the hydrophobic lipid bilayer of the bacterial cytoplasmic membrane (3). At physiological pH tetracyclines can exist as an equilibrium mixture of two free base forms: a low-energy, lipophilic nonionized species and a high-energy, hydrophilic zwitterionic structure (7). A solvent-dependent equilibrium between the two forms has been demonstrated with oxytetracycline free base and is supported by X-ray analyses of tetracyclines crystallized from aqueous and nonaqueous solvents (7, 15). Both forms are believed to be important for the antibacterial activity of tetracyclines, the low-energy, lipophilic conformational form (Fig. 1A) for uptake across the cytoplasmic membrane and the hydrophilic, zwitterionic structure (Fig. 1B) for binding to the ribosome (7).

Chelocardin (14) is a naturally occurring anhydrotetracycline derivative with a modified A ring (Fig. 2). In contrast to the solvent-dependent equilibrium of the two tetracycline species mentioned above, chelocardin apparently exists in the same conformation in both polar and nonpolar solvents, as evidenced by circular dichroism measurements (6). We believe this is related to the planarity of the BCD rings in chelocardin and that a lipophilic form, perhaps related to that of tetracycline, is the preferred species. These observa-

tions suggest that chelocardin interacts only poorly with ribosomes. Nevertheless, chelocardin is reported to be an effective inhibitor of protein synthesis and apparently has the same mechanism of action as chlortetracycline (9). Because of this apparent anomaly, we have reinvestigated the mode of action of chelocardin. In this study we show that the activities of chelocardin, other anhydrotetracyclines, and 6-thiatetracycline do not result from direct inhibition of protein synthesis at the level of the ribosome. In contrast to other tetracyclines, the antibacterial activity of these compounds may result from their ability to damage directly the cytoplasmic membrane.

MATERIALS AND METHODS

Chemicals. Chelocardin and 6-thiatetracycline were gifts from, respectively, Abbott Laboratories, North Chicago, Ill., and E. Merck, Pharmaceutical Research, Darmstadt 1, Germany. Other tetracyclines were prepared in Lederle Laboratories. The following radioactive chemicals were purchased from New England Nuclear Research Products, Boston, Mass.: L-[³⁵S]methionine (>1,000 Ci/mmol), [³H]thymidine (81.9 Ci/mmol), [5,6-³H]uridine (36.5 Ci/mmol), and L-U-¹⁴C-labelled amino acid mixture. All other chemicals were purchased from standard commercial sources.

Bacterial strains and plasmids. Bacteria and plasmids used in this study are described in Table 1.

Growth media. *Escherichia coli* strains were cultured in M63 minimal medium prepared as previously described (10) and supplemented when appropriate for the specific auxotrophic requirements of the strain. *Bacillus subtilis* was cultured in LB (10).

MIC determinations. MICs were determined by using an

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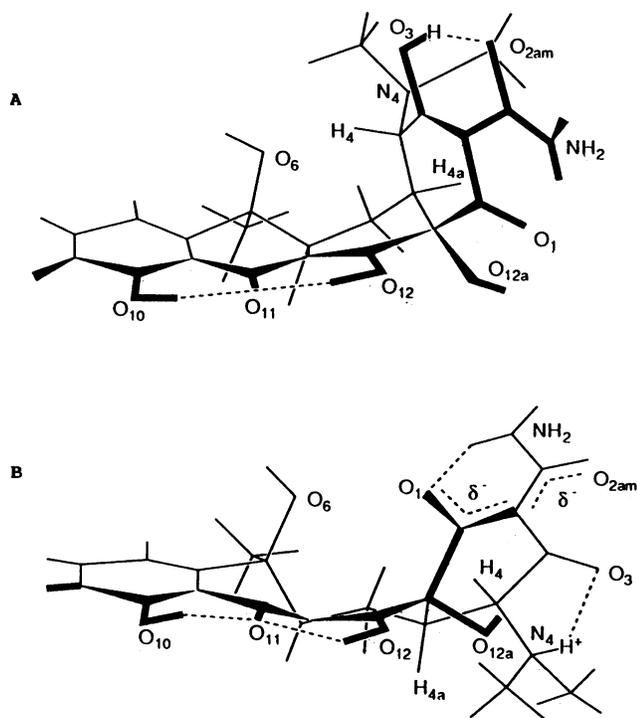


FIG. 1. Conformations of tetracycline free base species: lipophilic nonionized form (A) and polar zwitterionic form (B). Reproduced, with modifications, from Rogalski (15).

agar dilution method. A log-phase culture ($10 \mu\text{l}$) containing 1×10^6 to 5×10^6 CFU/ml was inoculated onto agar plates containing a range of antibiotic concentrations. Plates were incubated for 18 h at 37°C . The MIC was defined as the lowest concentration of antibiotic that inhibited growth of the organism.

In vitro translation systems. Cell-free translation systems from *E. coli* and *B. subtilis* were prepared and used essentially as previously described (5, 18). The *E. coli* system involved coupled transcription-translation with plasmid pBR322 as a template, whereas polypeptide synthesis in the *B. subtilis* system was directed by endogenous mRNA. The level of translation was measured by the incorporation of [^{35}S]methionine into trichloroacetic acid (TCA)-precipitable material. Reaction mixture ($2.5 \mu\text{l}$) was mixed with 0.5 ml of 1 N NaOH . After 15 min at 37°C , 2 to 3 ml of 25% TCA-0.1% Casamino Acids was added. Precipitated material was collected on Whatman GF/C filters and washed with 10% TCA followed by EtOH. The filters were dried at room temperature. Radioactivity retained on the filters was counted in a Beckman LS 7800 liquid scintillation counter.

Labelling of macromolecules in whole cells exposed to tetracyclines. Tetracyclines ($10 \mu\text{g/ml}$, final concentration) were added to cultures of *E. coli* MC 4100 (approximately 10^8 cells per ml) growing exponentially at 37°C in M63 liquid medium. Fifteen minutes later, 1-ml aliquots of these cultures were removed and labelled for 4.5 min with [$\text{methyl-}^3\text{H}$]thymidine ($1 \mu\text{Ci/ml}$), [$5,6\text{-}^3\text{H}$]uridine ($1 \mu\text{Ci/ml}$), or L- ^{14}C -labelled amino acid mixture ($0.25 \mu\text{Ci/ml}$). Incorporation was stopped by the addition of 1 ml of 10% (wt/vol) TCA and placement on ice. Incorporation of radiolabel into macromolecules was determined as described above. The levels of incorporation of the precursors into DNA, RNA,

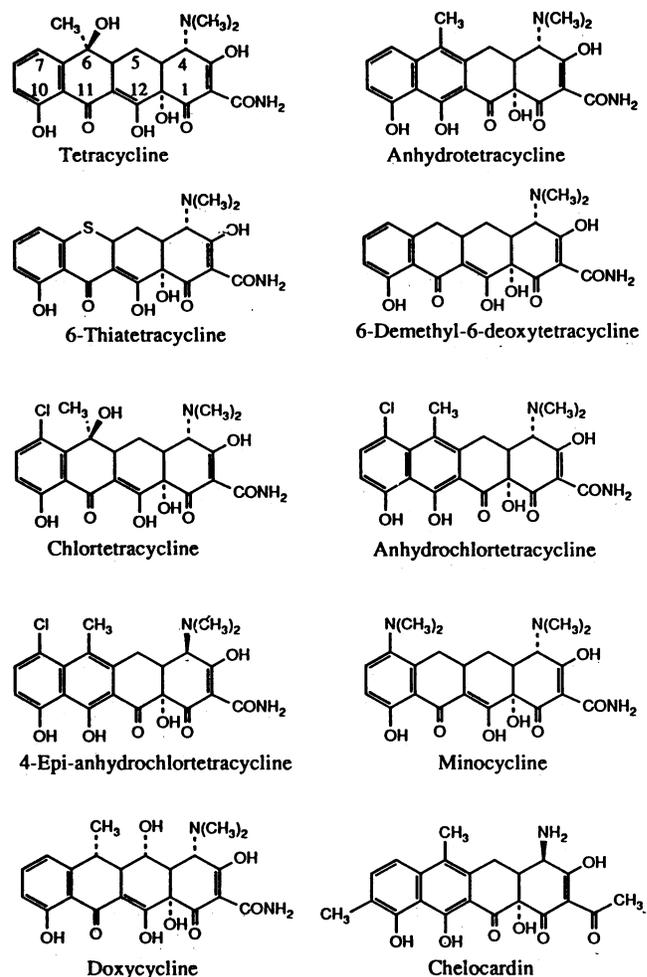


FIG. 2. Structure of tetracycline and some of its analogs.

and protein were compared with the incorporation levels in cultures lacking antibiotic and similarly labelled.

Induction of β -galactosidase in JC3272(pUB3610) by tetracyclines and determination of enzyme specific activity. The procedures used were as described previously (4).

Preparation of ribosomes and structural analysis of 16S rRNA. *E. coli* MRE600 0.5 M salt-washed ribosomes were

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
Strains		
<i>E. coli</i> MRE600	<i>rna</i>	1
<i>E. coli</i> K-12 MC4100	<i>araD139</i> Δ (<i>lac</i>) <i>U169</i> <i>strA thi</i>	2
<i>E. coli</i> K-12 JC3272	<i>his</i> Δ <i>lacX74 lys rpsL</i> <i>strA trp</i>	4
<i>B. subtilis</i> 168	<i>trpC2</i>	Bacillus Genetics Stock Center strain 1A1
Plasmids		
pBR322	Amp ^r <i>tetA</i>	4
pUB3610	Amp ^r <i>tetA-lacZ tetR</i>	4

TABLE 2. Susceptibility of *E. coli* K-12 MC4100 and *B. subtilis* 168 to tetracyclines

Antibiotic	MIC ^a (μg/ml)	
	<i>E. coli</i>	<i>B. subtilis</i>
Tetracycline	0.5	1.0
Chlortetracycline	0.25	0.5
Minocycline	0.25	<0.125
Doxycycline	0.25	<0.125
6-Demethyl-6-deoxytetracycline	0.5	<0.125
Chelocardin	0.5	0.125
Anhydrotetracycline	2.0	0.5
Anhydrochlortetracycline	4.0	0.5
4-Epi-anhydrochlortetracycline	8.0	2.0
6-Thiatetracycline	0.5	<0.125

^a MIC determinations were performed as described in Materials and Methods.

prepared as described (14). Antibiotics (500 μM) were incubated with 20 pmol of 70S ribosomes in 50 μl of 80 mM potassium cacodylate (pH 7.2) containing 20 mM MgCl₂, 100 mM NH₄Cl, 1 mM dithiothreitol, and 0.5 mM EDTA for 30 min at 37°C and then for 10 min at room temperature. Chemical modification was performed by addition of dimethyl sulfate (DMS) (2 μl of a 1:10 dilution in 95% ethanol) followed by incubation at 37°C for 6 min. The reactions were stopped, and the RNA was extracted as previously described (11). Primer extension and gel electrophoresis were performed as described previously (17).

RESULTS

Effects of tetracyclines on bacterial growth. The tetracyclines studied here (Fig. 2) were all effective inhibitors of bacterial growth, and the majority displayed MICs of ≤1 μg/ml against *E. coli* or *B. subtilis* (Table 2).

Effects of tetracyclines on protein synthesis directed by cell-free systems. The tetracyclines studied could be grouped into two broad categories on the basis of their activities as inhibitors of protein synthesis directed by an *E. coli* cell-free translation system.

Tetracycline, chlortetracycline, minocycline, doxycycline, and 6-demethyl-6-deoxytetracycline (Fig. 2) composed a group of effective protein synthesis inhibitors (Table 3). These compounds caused essentially complete inhibition of protein synthesis at 1.0 μg/ml, with substantial inhibition (>70%) at 0.3 μg/ml (Table 3). The second group of compounds comprised chelocardin, anhydrotetracycline, anhydrochlortetracycline, 4-epi-anhydrochlortetracycline, and 6-thiatetracycline (Fig. 2). These compounds were poor inhibitors of protein synthesis, causing <65% inhibition at concentrations of 1.0 μg/ml and <30% at 0.3 μg/ml (Table 3).

Representative members of the two groups of compound were also tested in a *B. subtilis* cell-free translation system (Table 3). Tetracycline, chlortetracycline, minocycline, and doxycycline inhibited protein synthesis at concentrations between 0.1 and 1.0 μg/ml, whereas chelocardin, anhydrotetracycline, anhydrochlortetracycline, and 6-thiatetracycline failed to inhibit protein synthesis even at 1.0 μg/ml (Table 3).

Effects of tetracyclines on the incorporation of precursors into macromolecules in *E. coli*. Results described in previous sections show that a number of tetracycline analogs (partic-

TABLE 3. Effects of tetracyclines on cell-free translation

Antibiotic	Concentration (μg/ml)	% Inhibition of translation ^a	
		<i>E. coli</i>	<i>B. subtilis</i>
Tetracycline	1	98	77
	0.3	69	ND
	0.1	ND	70
Chlortetracycline	1	100	85
	0.3	94	ND
	0.1	ND	81
Minocycline	1	100	47
	0.3	85	ND
	0.1	ND	47
Doxycycline	1	77	67
	0.3	70	ND
	0.1	ND	46
6-Demethyl-6-deoxytetracycline	1	100	ND
	0.3	90	ND
	0.1	ND	ND
Chelocardin	1	33	0
	0.3	24	ND
	0.1	ND	0
Anhydrotetracycline	1	64	0
	0.3	31	ND
	0.1	ND	0
Anhydrochlortetracycline	1	4	0
	0.3	0	ND
	0.1	ND	0
4-Epi-anhydrochlortetracycline	1	60	ND
	0.3	0	ND
	0.1	ND	ND
6-Thiatetracycline	1	22	0
	0.3	0	ND
	0.1	ND	0

^a Inhibition (after 30 min) of [³⁵S]methionine incorporation into TCA-precipitable material compared with that in a sample incubated without antibiotic. ND, not determined.

ularly those in group 2) inhibit bacterial growth yet fail to inhibit protein synthesis directed by cell-free systems. The antibacterial activity of these compounds cannot therefore be ascribed to direct inhibition of protein synthesis. To elucidate this, we examined the effects of various tetracyclines on the incorporation of radiolabelled DNA, RNA, and protein precursors into macromolecules.

Tetracycline derivatives (10 μg/ml) were added to exponentially growing cultures of *E. coli* MC4100. Fifteen minutes postaddition, aliquots of these cultures were labelled with radioactive thymidine, uracil, or amino acids. The levels of incorporation of these precursors into DNA, RNA, and protein were compared with incorporation levels in cultures lacking antibiotic and similarly labelled. Tetracycline caused only slight inhibition of precursor incorporation into DNA and RNA (Fig. 3). However, it caused essentially complete inhibition of amino acid incorporation into protein (Fig. 3). Like tetracycline, doxycycline, chlortetracycline, and minocycline also caused preferential inhibition of protein synthesis, with little effect on DNA and RNA synthesis

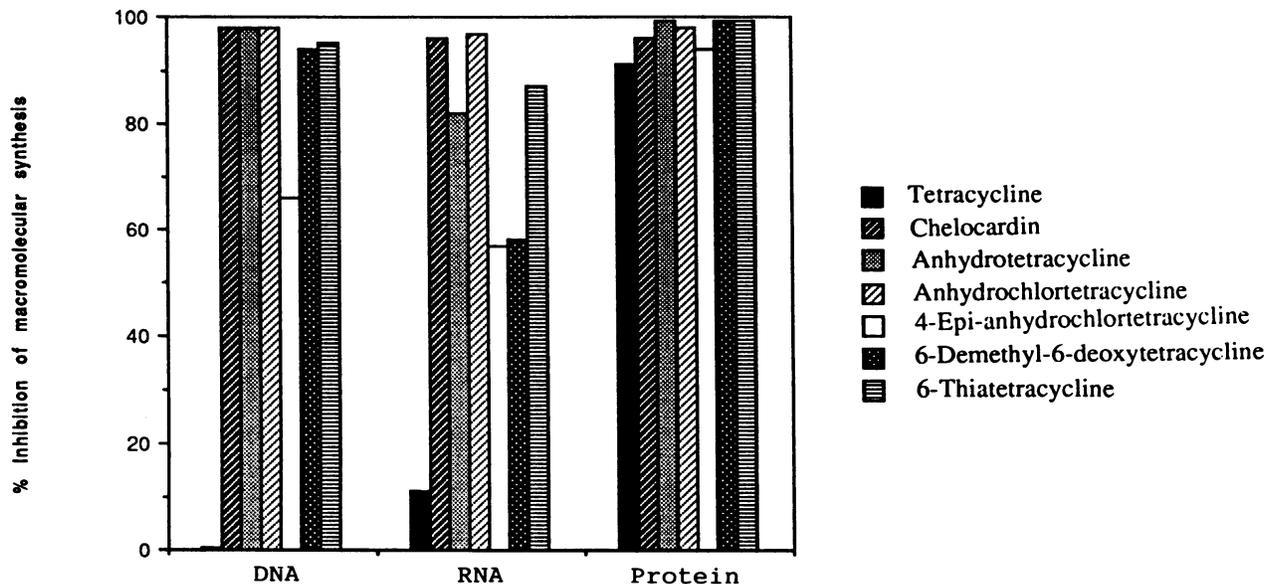


FIG. 3. Effects of tetracycline and some of its analogs on incorporation of radioactive precursors into DNA, RNA, and protein. Antibiotics (10 µg/ml) were added to cultures of *E. coli* K-12 MC4100 growing exponentially in M63 medium. Fifteen minutes later they were labelled with radioactive thymidine, uracil, or amino acids, and the incorporation of these precursors into DNA, RNA, and protein was determined as described in Materials and Methods. The results are expressed as percent inhibition of incorporation compared with that in a drug-free control culture.

(data not shown). In contrast to these tetracyclines, the other analogs exhibited substantial or complete inhibition of precursor incorporation into macromolecules, including nucleic acids (Fig. 3).

Entry of tetracyclines into the cytoplasm. Tetracycline analogs that inhibit macromolecular synthesis might exert their effect by interaction with the bacterial cell surface. Therefore it was of interest to determine whether these analogs were able to enter the cytoplasm. Entry of tetracyclines into the cytoplasm can be assessed by examining β-galactosidase induction in a strain carrying a *tetA-lacZ* fusion, e.g., pUB3610 in which expression of β-galactosidase from a *tetA-lacZ* translational fusion is controlled by the pSC101 *tetR* gene (4). Thus, induction of β-galactosidase synthesis by a tetracycline derivative reflects its entry into the cell and inactivation of the cytoplasmically located repressor encoded by *tetR*.

All the tetracyclines studied here induced β-galactosidase synthesis. For example, incubation of JC3272(pUB3610) in the presence of 25 to 100 ng of 6-demethyl-6-deoxytetracycline per ml led to enzyme induction (Table 4). The induction ratios obtained were comparable to those achieved when tetracycline was used as an inducer (Table 4).

Effects of tetracyclines on reactivity of bases in 16S rRNA to DMS. Antibiotics that interact directly with, or otherwise perturb, the structure of 16S rRNA change the chemical

reactivity of certain bases in the molecule to DMS (12). Previously, it was shown that chlortetracycline (Fig. 2) strongly protects A892 and causes enhanced reactivity of bases U1052 and C1054 in 16S rRNA to DMS (12), but the ability of other tetracycline analogs to affect the reactivity of bases in 16S rRNA was not tested. Since several tetracyclines were poor inhibitors of protein synthesis (Table 3), it was of particular interest to know whether they altered base reactivity to DMS.

Three patterns were observed: (i) compounds that protected A892 and stimulated reactivity of U1052 and C1054, e.g., chlortetracycline (Fig. 4) and tetracycline (data not shown); (ii) analogs that failed to protect A892, but stimulated reactivity of U1052 and C1054, e.g., anhydrochlortetracycline (Fig. 4), minocycline, doxycycline, 6-demethyl-6-deoxytetracycline, anhydrochlortetracycline, and 6-thiatetracycline (data not shown); and (iii) agents that failed to protect A892 or stimulate U1052/C1054, e.g., 4-epi-anhydrochlortetracycline and chelocardin (Fig. 4). Tetracycline derivatives in category i were active inhibitors of protein synthesis at the level of the ribosome, whereas those in category iii were not direct inhibitors of translation (Table 3). However, group ii contains some compounds that inhibited *in vitro* protein synthesis and others that did not (Table 3).

TABLE 4. Effects of antibiotics on induction of β-galactosidase in *E. coli* K-12 JC3272(pUB3610)

Antibiotic	Induction ratio ^a after incubation with antibiotic at concn (ng/ml) of:			
	25	50	75	100
Tetracycline	9.54 ± 2.35	12.85 ± 0.42	14.29 ± 0.79	12.45 ± 2.16
6-Demethyl-6-deoxytetracycline	7.07 ± 0.07	11.99 ± 1.06	12.08 ± 0.66	14.54 ± 0.43

^a Enzyme specific activity in the presence of drug divided by the basal (noninduced) activity. Induction ratios ± 1 standard deviation of the mean are the means of replicate determinates for each culture.

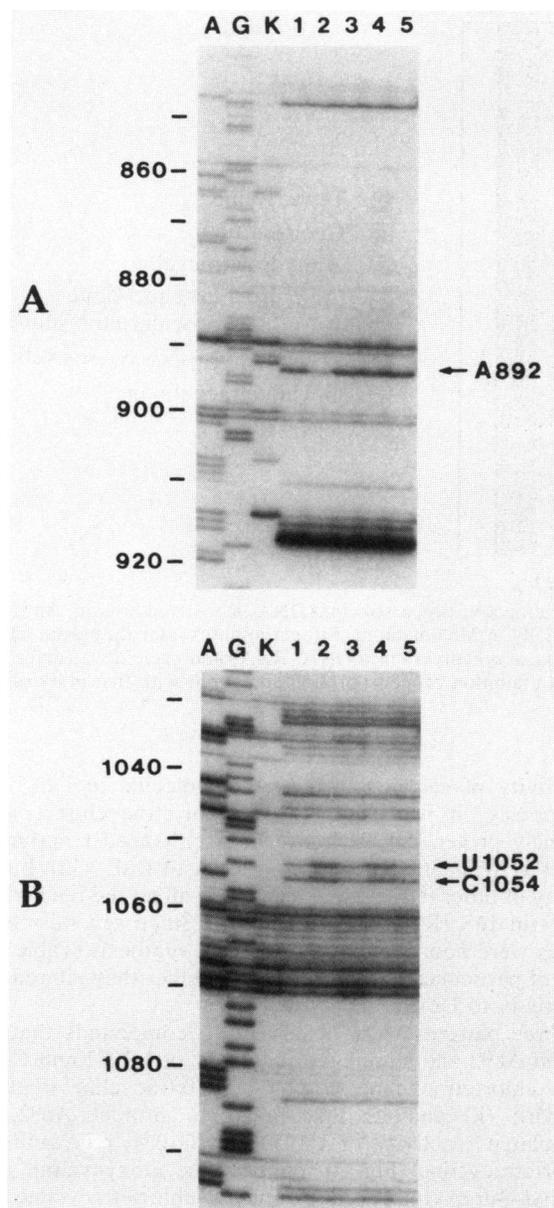


FIG. 4. Effects of tetracycline analogs on modification of bases in 16S rRNA. *E. coli* 70S ribosomes were incubated with 500 μ M drug as described in Materials and Methods. Sites of modification were detected by primer extension as previously described (17). Lanes AG, dideoxy sequencing lanes; lanes K, unmodified RNA, showing spontaneous stops; lanes 1, no drug; lanes 2, chlortetracycline; lanes 3, chelocardin; lanes 4, anhydrochlortetracycline; lanes 5, 4-epi-anhydrochlortetracycline. Arrows indicate protection of A892 (A) and enhancement of U1052 and C1054 (B) by certain drugs.

DISCUSSION

In agreement with earlier findings (3) we noted that tetracycline, chlortetracycline, and minocycline were effective inhibitors of protein synthesis directed by cell-free systems. We also established that other tetracyclines, e.g., doxycycline and 6-demethyl-6-deoxytetracycline, also inhibited cell-free translation. However, a group of tetracyclines

comprising chelocardin, anhydrotetracycline, anhydrochlortetracycline, 4-epi-anhydrochlortetracycline, and 6-thiatetracycline were poor inhibitors of in vitro protein synthesis.

We identified chelocardin as one of the tetracycline analogs which lacks direct inhibitory activity at the ribosome. However, this compound is reported to inhibit protein synthesis by the same mechanism as chlortetracycline (9). In addition to the data presented here showing that chelocardin does not inhibit cell-free translation, its failure to alter reactivity of bases in 16S rRNA to DMS is consistent with failure to interact with the ribosome. We have no explanation for the discrepancy between our results and the previous data claiming that chelocardin inhibits protein synthesis (9).

The properties of chelocardin are not unique amongst the tetracyclines, since anhydrotetracycline, anhydrochlortetracycline, 4-epi-anhydrotetracycline, and 6-thiatetracycline are also poor inhibitors of in vitro protein synthesis (Table 3) yet display antibacterial activity (Table 2). Therefore, the antibacterial activity of chelocardin and several other tetracycline analogs cannot be ascribed to direct inhibition of protein synthesis at the level of the ribosome. In whole bacteria these compounds caused rapid inhibition of incorporation of precursors into macromolecules. In contrast, tetracycline, doxycycline, chlortetracycline, and minocycline primarily prevented incorporation of amino acids into protein, which is in agreement with earlier findings (3).

The mechanism by which some of the tetracyclines prevent macromolecular synthesis in whole cells is of interest. Analogs exhibiting this activity are able to enter the cell because they induce β -galactosidase synthesis in strains carrying *tetA-lacZ* fusions. Although these tetracycline analogs do enter the cell, the ability of these compounds to inhibit multiple macromolecular synthetic processes simultaneously strongly suggests that they interfere with cytoplasmic membrane function. Inhibition of precursor incorporation into macromolecules is consistent with membrane perturbation which prevents substrate accumulation and leads to loss of essential cofactors from the cell (8, 16). These analogs may interfere with cytoplasmic membrane function, since inhibition of precursor incorporation into macromolecules is consistent with membrane perturbation which prevents substrate accumulation and leads to loss of essential cofactors from the cell (8, 16). Therefore, the antimicrobial activity of analogs that inhibit macromolecular synthesis may result from retention of a high proportion of these tetracycline molecules in the cytoplasmic membrane which suggests that antibiotic molecules entering the cytoplasm do not contribute to inhibition of bacterial growth. The ability of certain tetracyclines to act in this manner probably relates to the conformation they adopt, i.e., primarily lipophilic nonionized forms (see Introduction). In contrast, tetracycline, chlortetracycline, minocycline, and doxycycline, which are able to predominantly adopt hydrophobic or predominantly hydrophilic forms depending upon their environment, are probably not retained at the cell surface and are able to interact with ribosomes in the cell. We are currently pursuing the hypothesis that tetracyclines which stop precursor incorporation into macromolecules do so primarily by promoting membrane damage.

6-Demethyl-6-deoxytetracycline inhibits cell-free translation and prevents incorporation of precursors into macromolecules. The question of whether this compound inhibits protein synthesis in whole cells by direct interaction with ribosomes, by secondary effects, or a combination of both therefore arises. Induction of β -galactosidase by the com-

pound in the *tetA-lacZ* fusion strain indicates that it can enter the cytoplasm to interact with the *tet* regulatory elements and therefore probably also with the ribosome. However, 6-demethyl-6-deoxytetracycline also prevents macromolecular synthesis, so inhibition of protein synthesis in bacteria may reflect a dual mode of action.

Recently, DMS probing methods have been applied to analyze the interaction of chlortetracycline with ribosomes. This antibiotic protects A892 in 16S rRNA from DMS modification and enhances reactivity of U1052 and C1054 (12). However, the effects of other tetracyclines on the chemical reactivity of bases in 16S rRNA to DMS have not been reported. It was therefore of particular interest to examine those tetracyclines that were poor inhibitors of cell-free translation in order to determine whether the probing patterns differed from those obtained with effective ribosomal inhibitors. Three probing patterns were observed: (i) protection of A892 and stimulation of U1052/C1054; (ii) lack of A892 protection, but stimulation of U1052/C1054; and (iii) neither A892 protection nor U1052/C1054 stimulation. Compounds in category i inhibited protein synthesis directly, whereas derivatives in category iii were not ribosomal inhibitors and those in category ii contained both ribosomal and nonribosomal inhibitors.

These observations permit limited structure-activity correlations with respect to ribosomal binding and ribosomal inhibition. Although chlortetracycline and tetracycline protect A892, the ribosomal interaction leading to this response is not directly correlated to inhibition of protein synthesis since several effective ribosomal inhibitors (e.g., minocycline, doxycycline, and 6-demethyl-6-deoxytetracycline) do not protect A892. Examination of tetracycline structures (Fig. 2) indicates that the ability to protect A892 probably relates to possession of a pseudoaxial OH group at C-6. Chelocardin and 4-epi-anhydrochlortetracycline are not ribosomal inhibitors, nor do they alter reactivity of A892, U1052, or C1054 to DMS. These features are likely related to substitution at C-4 with moieties in the epi(β) configuration (Fig. 2). Finally, there is a group of tetracyclines which alter reactivity of U1052 and C1054 to DMS but are not necessarily effective ribosomal inhibitors. The affinity of these tetracyclines for the ribosome may be sufficient to alter chemical reactivity of bases in 16S rRNA to DMS but not necessarily adequate in all cases to prevent protein synthesis. Clearly, further studies are required to establish the exact relationship between 16S rRNA probing patterns and inhibition of protein synthesis by members of the tetracycline group.

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REFERENCES

1. Cammack, K. A., and H. E. Wade. 1965. The sedimentation behavior of ribonuclease-active and -inactive ribosomes from bacteria. *Biochem. J.* **96**:671-680.
2. Casadaban, M. J. 1976. Transposition and fusion of the *lac* operon to selected promoters in *Escherichia coli* using bacteriophage λ and Mu. *J. Mol. Biol.* **104**:541-555.
3. Chopra, I. 1985. Mode of action of the tetracyclines and the nature of bacterial resistance to them, p. 317-392. In J. J. Hlavka and J. H. Boothe (ed.), *Handbook of experimental pharmacology*, vol. 78. Springer-Verlag KG, Berlin.
4. Chopra, I., K. Hacker, Z. Misulovin, and D. M. Rothstein. 1990. Sensitive biological detection method for tetracyclines using a *tetA-lacZ* fusion system. *Antimicrob. Agents Chemother.* **34**:111-116.
5. Collins, J. 1979. Cell-free synthesis of proteins coding for mobilisation functions of ColE1 and transposition functions of *Tn3*. *Gene* **6**:29-42.
6. Ding, W.-D., and G. A. Ellestad. Unpublished data.
7. Hughes, L. J., J. J. Stezowski, and R. E. Hughes. 1979. Chemical structural properties of tetracycline derivatives. 7. Evidence for the coexistence of the zwitterionic and nonionized forms of the free base in solution. *J. Am. Chem. Soc.* **101**:7655-7657.
8. Konisky, J. 1982. Colicins and other bacteriocins with established modes of action. *Annu. Rev. Microbiol.* **36**:125-144.
9. Mao, J. C. H., and E. E. Robishaw. 1971. Mode of action of β -chelocardin. *Biochim. Biophys. Acta* **238**:157-160.
10. Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
11. Moazed, D., and H. F. Noller. 1986. Transfer RNA shields specific nucleotides in 16S ribosomal RNA from attack by chemical probes. *Cell* **47**:985-994.
12. Moazed, D., and H. F. Noller. 1987. Interaction of antibiotics with functional sites in 16S ribosomal RNA. *Nature (London)* **327**:389-394.
13. Moazed, D., B. J. Van Stolk, S. Douthwaite, and H. F. Noller. 1986. Interconversion of active and inactive 30S ribosomal subunits is accompanied by a conformational change in the decoding region of 16S rRNA. *J. Mol. Biol.* **191**:483-493.
14. Oliver, T. J., J. F. Prokop, R. R. Bower, and R. H. Otto. 1962. Chelocardin, a new broad-spectrum antibiotic. I. Discovery and biological properties. *Antimicrob. Agents Chemother.* **583-591**.
15. Rogalski, W. 1985. Chemical modification of the tetracyclines, p. 179-316. In J. J. Hlavka and J. H. Boothe (ed.), *Handbook of experimental pharmacology*, vol. 78. Springer-Verlag KG, Berlin.
16. Sahl, H. G. 1985. Influence of the staphylococcin-like peptide Pep5 on membrane potential of bacterial cells and cytoplasmic membrane vesicles. *J. Bacteriol.* **162**:833-836.
17. Stern, S., D. Moazed, and H. F. Noller. 1988. Structural analysis of RNA using chemicals and enzymatic probing monitored by primer extension. *Methods Enzymol.* **164**:481-489.
18. Zubay, G. 1973. In vitro synthesis of protein in microbial systems. *Annu. Rev. Genet.* **7**:267-287.