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Mode of Action

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Edited by
H. SCHÖNFELD, Grenzach and A. DE WECK, Bern

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Vol.11: 2nd International Symposium of Chemotherapy/2. Internationales Symposium für Chemotherapie/2e Symposium international de chimiothérapie, Naples 1961. Part I: New Antibiotics – Neue Antibiotica – Nouveaux antibiotiques. Side effects of Modern Chemotherapy – Nebenwirkungen der modernen Chemoterapeutica – Effects secondaires des chimiothérapies modernes. XXII+354 p., 54 fig., 85 tab., 1963. sFr. 78.—.

Vol.12: Pharmakokinetik und Arzneimitteldosierung/Pharmacokinetics and Drug Dosage. Kolloquium im Forschungsinstitut Borstel 1962. Hgb. von/edited by: ENNO FREERKSEN (Borstel), unter Mitarbeit von/with the collaboration of L. DETTLI (Basel), E. KRÜGER-THIEMER (Borstel), E. NELSON (Buffalo, N.Y.). XVI+455 p., 161 fig., 46 tab., 1964. sFr. 70.—.

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Vol.15: The immune Response and its Suppression. An International Symposium held at the Forschungsinstitut Davos (1968). VIII+422 p., 148 fig., 64 tab., 1969. sFr. 98.—.

Vol.16: Experimental and Clinical Evaluation of the Tuberculostatics Capreomycin, Isoxyl, Myambutol, Rifampicin. International Colloquy, Borstel (Germany) 1968. Editor: H.-J. THUMIM (Borstel, Germany). X+536 p., 150 fig., 251 tab., 1970. sFr. 145.—.

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Foreword

'What we want is a Chemotherapia specifica, that is, we are looking for agents, that on the one hand are able to kill certain parasites, without on the other hand causing too much harm to the organism when applied in doses necessary to kill these parasites.'

With these words spoken at a meeting of the Berlin Medical Society on the 13th February, 1907 PAUL EHRLICH opened up a path on which we have come far but whose end – that is the killing of parasites without harming the patient – is still a long way off. Only very few antibiotics and chemotherapeutics develop bactericide, i.e. germ killing properties, and with none of them are side effects excluded. The only solution open to us is to assess the chances of success against risk and try to determine in each case the most efficient dose.

To achieve this we need a subtle knowledge of drugs. Of course this is not only true in the field of chemotherapy; however in our branch we are faced with the curious situation that we administer a medicine to a patient, which is not intended for the patient but for an organism within him. Therefore all direct effects on the patient must be side effects.

We believe that the side effects of antibiotics and chemotherapeutics belong to the problems which should be considered when choosing the preparation, for example the danger of the diffusion of toxins through destruction of the cell membranes during penicillin treatment of say Typhus abdominalis. We have therefore undertaken in the present volume to illustrate the mode of action of a number of antibacterially effective substances. We thought it not necessary to discuss all groups of antibiotics as outstanding accounts, for example of penicillin by STROMINGER and chloramphenicol by HAHN exist.

This volume about the mode of action of antibacterially efficient substances signals a change in the style of our series, in that in future every volume will deal with a specific subject, whereby we are thinking of introducing original work alongside accounts.

Finally we have decided to rename ANTIBIOTICA ET CHEMOTHERAPIA, an already traditional series, ANTIBIOTICS AND CHEMOTHERAPY and to present in future articles only in the English language, which should not be a limitation but should contribute towards improving the international communication of scientific results.

HANS SCHÖNFELD

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Antibiotics and Chemotherapy, vol. 17, pp. 1–28 (Karger, Basel 1971)

Tetracyclines

ALLEN I. LASKIN and JEROLD A. LAST

Esso Research and Engineering Co., Linden, N. J. and
Rockefeller University, New York, N. Y.

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I. Introduction

The mechanism of action of the tetracycline antibiotics was the subject of two recent extensive review articles [17, 30]. Some of the later studies were covered as part of a symposium on molecular aspects of antibiotic activity [31], and were included in a general review of antibiotics that act upon the bacterial ribosome [63]. In addition, some of the pertinent literature through early 1969 was discussed in a comprehensive review on the mechanism of protein biosynthesis [37b]. In all of these publications it was concluded that the most important inhibitory effect of tetracyclines, when they were tested at bacteriostatic concentrations, was inhibition of protein biosynthesis. The present paper will review the newer evidence that tetracyclines act at the bacterial ribosome to inhibit protein biosynthesis, and will attempt to identify

more precisely the reactions sensitive to these antibiotics. Some of the more recent papers related to the mechanism of resistance of bacteria to the tetracyclines will also be discussed.

II. Mechanism of Action

A. Binding of Aminoacyl-tRNA to Ribosomes

It has been suggested that there are two sites on the 70S ribosome that are able to bind aminoacyl-tRNA [5]. Reports that tetracyclines inhibited the binding of aminoacyl-tRNA to ribosomes by no more than about 50% [21, 58] led to the suggestion that these antibiotics blocked one of the two postulated binding sites for aminoacyl-tRNA [58]. Many investigators have attempted to determine more exactly the nature and location on the ribosome of the binding site sensitive to tetracyclines.

LUCAS-LENARD and HAENNI [39] reported that chlortetracycline strongly inhibited the binding of phenylalanyl-tRNA to ribosomes that had previously been allowed to bind N-acetylphenylalanyl-tRNA. In this system, binding of phenylalanyl-tRNA is dependent upon the addition of guanosine triphosphate (GTP) and the binding 'enzyme', T [20]. The 'enzyme' T is actually a partially purified fraction which contains two enzymes, one of which is designated T_s (stable) and the other T_u (unstable). The binding of aminoacyl-tRNA to the ribosome has been shown to be a complicated process. In the first step, T_s catalyzes the formation of a T_u-GTP complex. Then, aminoacyl-tRNA reacts with this complex to form an aminoacyl-tRNA-T_u-GTP complex that is an intermediate in the binding of aminoacyl-tRNA to ribosomes [46, 50, 64].

The effect of deacylated-tRNA (transfer RNA not charged with its cognate amino acid) on the binding of phenylalanyl-tRNA to ribosome-polyuridylic acid complexes was investigated by CONWAY and his co-workers [56]. They found that crude, uncharged transfer RNA antagonized the binding of equivalent amounts of phenylalanyl-tRNA. A combination of tetracycline and deacylated-tRNA gave additive inhibition of phenylalanyl-tRNA binding. They concluded that deacylated-tRNA inhibited binding of phenylalanyl-tRNA at one site and that tetracycline inhibited phenylalanyl-tRNA binding at a different site [56]. These workers suggested that the site sensitive to inhibition by tetracycline was the A site (also referred to as the aminoacyl-tRNA site, the amino acid site, the decoding site, and the entering site by

different authors), while the site sensitive to inhibition by deacylated-tRNA was the P site (also referred to as the peptidyl site, the leaving site, and the puromycin-sensitive site by different authors). In a subsequent study, ribosomes were treated with N-ethylmaleimide (a reagent that probably reacts with sulfhydryl groups on ribosomal proteins) to reduce their capacity for the binding of phenylalanyl-tRNA and polymerization of phenylalanine; 50S and 30S subunits from treated ribosomes did not associate to 70S ribosomes except at high concentrations of magnesium acetate (20 mM, compared with 8 mM routinely used for this association). The amount of phenylalanyl-tRNA bound to these ribosomes (treated with N-ethylmaleimide) was measured. Both deacylated-tRNA and tetracycline inhibited binding to the same extent as with untreated ribosomes; the inhibition of binding by a mixture of the two inhibitors was still approximately additive. It was concluded that N-ethylmaleimide, which inhibited phenylalanyl-tRNA binding by 66% (in the presence of a concentration of 8 mM Mg^{++}) did not act preferentially with respect to either the A or the P site on the ribosome [51].

KAJI and his co-workers reported that tetracycline inhibited the binding of phenylalanyl-tRNA to 30S ribosomal subunits by 70% [60]. When 50S subunits were added to the 30S subunits, 70S ribosomes were reconstituted; at the same time, the binding capacity for phenylalanyl-tRNA doubled (50S subunits alone did not bind phenylalanyl-tRNA). Tetracycline completely inhibited the binding of the second molecule of phenylalanyl-tRNA to the newly-formed binding site when 30S and 50S subunits were combined [60]. The 30S ribosomal site corresponds to the P site; the 'newly-formed' site on the 70S ribosome corresponds to the A site [23, 24]. Once phenylalanyl-tRNA was bound to 30S ribosomal subunits or to 70S ribosomes, tetracycline was not able to dissociate the complex [60].

Other aspects of the binding of aminoacyl-tRNA to ribosomes that are sensitive to inhibition by the tetracyclines have also been reported by KAJI's group. Specific (messenger RNA-dependent) binding of aminoacyl-tRNA to ribosomes was much more sensitive to inhibition by tetracycline than was non-specific binding [61]. The transfer RNA that is capable of being charged with phenylalanine (that is, deacylated phenylalanyl-tRNA) was released from the ribosome-polyuridylic acid-phenylalanyl-tRNA complex, after transfer of phenylalanine into peptide linkage, in a reaction that required GTP. This release reaction was inhibited by about 70% by tetracycline [29]. This release of spent transfer RNA was dependent on transfer factor G [KAJI, personal communication] and probably represented a partial reaction of translocation. The term translocation in this context refers to the movement of the ribo-

some, relative to messenger RNA, that allows the sequential reading of the information encoded in the messenger. Translocation is dependent upon GTP and G enzyme. Finally, IGARISHI and KAJI [24] reported that a high concentration of tetracycline (5×10^{-4} M) preferentially (by about a 2:1 ratio) inhibited the binding of phenylalanyl-tRNA to the A site (rather than to the P site). At a concentration of 5×10^{-5} M tetracycline, however, the binding of phenylalanyl-tRNA to both the A and the P sites was inhibited to an equal extent. This latter concentration of tetracycline (5×10^{-5} M) was sufficient to inhibit the binding of phenylalanyl-tRNA to ribosomes, under their standard assay conditions, by about 50% [24]. It is interesting to note that under their standard assay conditions, at a concentration of 5 mM Mg^{++} ion, about 75% of the phenylalanyl-tRNA bound is in the P site; however, when the Mg^{++} concentration is raised to 13 mM, both the A and the P site are equally occupied by phenylalanyl-tRNA.

GOTTESMAN [18] reported that chlortetracycline inhibited the addition of lysyl-tRNA to a polylysyl-tRNA-ribosome-polyadenylic acid complex. The formation of polylysylpuromycin was not inhibited by chlortetracycline. This result was interpreted as due to a specific inhibition of the binding of lysyl-tRNA to the A site of the ribosome by chlortetracycline [18].

The effect of tetracycline on the GTP-dependent binding of N-formylmethionyl-tRNA to a complex of ribosomes and the trinucleotide AUG was studied by SARKAR and THACH [54]. At a concentration of 3×10^{-4} M, tetracycline inhibited the binding of N-formylmethionyl-tRNA to either 70S ribosomes or 30S ribosomal subunits by about 50%. These observations were interpreted as a specific inhibition of binding at the A site [54]. The results in this paper also demonstrated that the polyuridylic acid-dependent binding of phenylalanyl-tRNA was inhibited 60% by 1×10^{-4} M tetracycline. Essentially all of the phenylalanyl-tRNA bound in the presence of tetracycline reacted with puromycin; that is, was bound at the P site [see also ref. 24]; the second molecule of phenylalanyl-tRNA, which bound in the absence of tetracycline, was not reactive with puromycin; that is, was bound at the A site.

All of the studies discussed thus far have dealt with the effect of tetracyclines on the binding of aminoacyl-tRNA to 70S ribosomes or to ribosomal subunits. Since protein synthesis in growing bacteria (and other organisms) seems to take place on polysomes, which are aggregates of ribosomes held together by messenger RNA [45], we can ask whether the inhibition of aminoacyl-tRNA binding by tetracyclines also occurs on polysomes. MAXWELL prepared polysomes from lysates of *Bacillus megaterium* proto-

plasts [43]. This preparation actively incorporated amino acids into polypeptides; incorporation could be stopped rapidly and completely by about 2×10^{-4} M tetracycline. An experiment was performed with polysomes labeled with C^{14}-amino acids and P^{32}-transfer RNA. Analysis of such labeled polysomes by centrifugation through sucrose density gradients demonstrated that tetracycline did not affect the attachment of C^{14}-labeled peptides to the polysomes. However, about 30% of the P^{32}-labeled transfer RNA was removed from the polysomes after incubation of the bacteria with tetracycline. MAXWELL concluded that this loss of labeled transfer RNA represented a *specific* loss of aminoacyl-tRNA from the polysomes, while peptidyl-tRNA remained attached [43].

MODOLELL and DAVIS prepared polysomes by the addition of messenger RNA from the bacteriophage, R-17, to cell-free extracts prepared from *Escherichia coli* [45]. The addition of streptomycin caused a rapid breakdown of these polysomes to 70S ribosomes. Chlortetracycline alone, at about 2×10^{-4} M, had no effect on polysome integrity over the short interval of time in which the breakdown of polysomes induced by streptomycin occurred. However, chlortetracycline antagonized the breakdown of polysomes by streptomycin; in the presence of chlortetracycline, the breakdown induced by streptomycin took place at a slower rate. These results were interpreted in terms of a model in which streptomycin causes a 'distortion' of the A site on the ribosome sufficient for peptidyl-tRNA to be lost from the polysomes during its transfer from the P site to the A site. This loss of peptidyl-tRNA was postulated as the cause of the observed breakdown of the polysomes in these extracts. Chlortetracycline was assumed to block the transfer of peptidyl-tRNA out of the P site by blocking the binding of aminoacyl-tRNA into the A site, with a consequent stabilization of the polysomes [45].

To our knowledge, there has been no *direct* demonstration of an inhibition of the binding of aminoacyl-tRNA to polysomes by tetracyclines. For technical reasons, such an experiment would be difficult to perform. However, such an inhibition has been directly demonstrated with 70S ribosomes (monosomes) and the indirect evidence we have cited above suggests that the tetracycline antibiotics do indeed inhibit the binding of aminoacyl-tRNA to polysomes.

All of the studies described above are consistent with the idea that tetracycline antibiotics inhibit the binding of aminoacyl-tRNA to the A site on the 30S ribosomal subunit. The results of these studies are customarily interpreted in terms of a model [5] that proposes that there are two binding sites for transfer RNA on the ribosome. The A site binds an aminoacyl-tRNA

molecule, coded for by the specific triplet on the messenger RNA that is being translated, in a reaction requiring GTP and the enzymes T_s and T_u. Once this aminoacyl-tRNA is bound, the nascent peptidyl-tRNA, which is (already) bound in the P site, reacts with the aminoacyl-tRNA in the A site to make a new peptidyl-tRNA, one amino acid longer, in the A site. This reaction (peptide bond formation) is catalyzed by an enzyme that is an integral part of the 50S ribosomal subunit [41]. Finally, in a series of reactions requiring GTP and the enzyme G, the deacylated transfer RNA is expelled from the P site; the newly formed peptidyl-tRNA is translocated from the A site to the P site, and either the ribosomal sites or the messenger RNA move in such a way as to place the next triplet of the messenger to be translated into the A site.

It would be very satisfying to be able to stop at this point and to say that the mechanism of action of the tetracyclines has been elucidated. However, there are some serious inconsistencies with some of the observations discussed above, and the hypothesis derived from them.

SHOEMAKER and NOLL [57] reported that, contrary to the findings of GOTTESMAN [18] and of SARKAR and THACH [54], tetracycline did inhibit the polyadenylic acid-directed binding of polylysyl-tRNA to ribosomes. This observation may be explained most conveniently if it is postulated that any transfer RNA molecule may only gain access to the P site *via* translocation from the A site [54]. The evidence that polylysyl-tRNA was bound at the A site (at least initially) in the study of SHOEMAKER and NOLL was: (1) GTP or supernatant factors were not required for the binding of polylysyl-tRNA to the ribosomes; (2) GTP and supernatant *were* required to convert the bound polylysyl-tRNA to a state where it was able to react with puromycin to form polylysylpuromycin; (3) GTP and supernatant were also required to convert ribosomes that had already bound polylysyl-tRNA into a state where they were able to bind lysyl-tRNA; and (4) tetracycline blocked the binding of the polylysyl-tRNA in this system. No explanation was given for the discrepancies between these observations [57] and the earlier reports that the binding of polylysyl-tRNA to ribosomes was not sensitive to tetracyclines [18, 54].

SUAREZ and NATHANS first postulated that tetracycline inhibited the binding of aminoacyl-tRNA at one of two ribosomal sites in order to explain why the maximal inhibition that they observed did not exceed about 50% [58]. However, it is very difficult to draw valid conclusions about the exact maximum percentage of inhibition that is observed in this reaction. Small variations in the extent of binding between control samples and samples treated with antibiotic are magnified when results are compared on a per-

Table I. Inhibition of polyuridylic acid-directed binding of phenylalanyl-tRNA to *E. coli* ribosomes

Antibiotic added	Per cent inhibition by:			
	Tetracycline	Oxytetracycline	Chlortetracycline	Demethylchlortetracycline
μg/ml				
1	26	22	28	30
10	59	51	64	67
20	62	57	60	66
200	76	74	71	75
5	–	15	–	–
25	–	57	–	–
100	–	74	–	–

centage basis. With the customary specific activities of C^{14}-labeled aminoacyl-tRNA used in this assay, only a few counts per minute correspond to a difference of a few per cent inhibition observed. Nevertheless, it should be noted that tetracyclines have been reported to inhibit the binding of aminoacyl-tRNA to ribosomes by *more* than 50% in studies from a number of different laboratories. HIEROWSKI reported 60% inhibition of binding of phenylalanyl-tRNA by chlortetracycline [21]. KAJI found 70% inhibition by tetracycline in one study [60], and inhibitions in excess of 85% in another [24]. COHEN *et al.* reported a 72% inhibition of aminoacyl-tRNA binding by chlortetracycline [6]. We have previously reported inhibitions of the polyuridylic acid-directed binding of phenylalanyl-tRNA of 70 to 80% by oxytetracycline [37a] and of over 60% by tetracycline [33]. The polyadenylic acid-directed binding of lysyl-tRNA was inhibited up to 78% by tetracycline [33].

It may be argued that such high inhibitions of the binding of aminoacyl-tRNA to ribosomes as cited above occurred only in the presence of very high concentrations of the tetracycline antibiotics and were somehow artifactual. However, we have also observed inhibition of the binding reaction in excess of 50% with low to moderate concentrations of various tetracyclines as shown in table I [31; IZAKI and LAST, unpublished].