*INHIBITION OF PROTEIN SYNTHESIS BY CHLORTETRACYCLINE IN THE E. COLI IN VITRO SYSTEM**

By Marian Hierowski†

THE ROCKEFELLER INSTITUTE

Communicated by Fritz Lipmann, January 29, 1965

The antibiotic action of an increasing number of compounds appears to be due to an inhibition of protein synthesis. The requirement for a rapid protein synthesis in growing bacteria is much greater than in the mostly stationary mammalian cells. For example, inhibition by chloramphenicol is virtually absent with most mammalian systems, and even in bacteria is considerably more pronounced in a system where template RNA is added to the ribosomes than with ribosomes that carry their natural template.[1, 2] In general, most of the antibiotics that inhibit protein synthesis do so in an *in vitro* system composed of ribosome and supernatant fractions. The more we have become acquainted through *in vitro* studies with the finer mechanism of action of the various antibiotics, the more they have become available as tools in studying the stages of protein synthesis.

Although an inhibition of protein synthesis was suggested as a mode of action for tetracycline quite a while ago by Gale and Folkes,[3] and was shown more directly by Nikolov and Ilkov,[4] and by Rendi and Ochoa,[5] not much attention was paid to the mechanism of this inhibition until recently when Franklin published two careful studies,[6] showing inhibitory effects on microbial as well as mammalian systems by all the tetracyclines, but most pronouncedly by chlortetracycline. He found tetracycline to inhibit polypeptide synthesis in the Nirenberg-Matthaei system[7] using phenylalanyl sRNA and polyuridylic acid (poly U) on *E. coli* ribosomes; he also studied inhibition in rat liver preparations. He did not reach any definite conclusions as to the point of action of the antibiotic, but suggested that it was involved in the synthesis of the peptide bond, since he could show no inhibition with amino acid activation and transfer to sRNA.

In the context of the efforts of this laboratory to analyze the mechanism of peptide bond formation,[8, 9] a more detailed study of the mechanism of inhibition by tetracycline appeared desirable. The results to be reported here indicate tetracycline to belong in a group with chloramphenicol and streptomycin; their action appears to be due to either a modification of or an interference with the assembling of the components of the synthetic system on the ribosome. The main interference by tetracycline seems to be localized in binding of amino acyl sRNA to the ribosome. Such an interference with amino acyl sRNA binding has been independently observed by Suarez and Nathans.[10]

Methodology.—Extracts of alumina-ground frozen cells of *E. coli* B were used. They were prepared as described in previous publications from this laboratory using the procedure of Nirenberg and Matthaei.[7] Frequently, their S-30 preparation was used, that is, the supernatant obtained after centrifugation for 30 min at 30,000 $\times$ g. The preparation was mostly depleted of its capacity for endogenous synthesis by preliminary incubation. The fraction was stored at $-20°$. Ribosomes were isolated from this fraction as described,[11] washed twice with 0.01 M Tris buffer pH 7.4 and 0.01 M $MgCl_2$ solution, and stored frozen.

Preparation of sRNA and its charging with amino acids was carried out as described.[11] With the amino acyl-sRNA-ribosome system, the transfer fraction described by Conway and Lipmann[12] was used. In the experiments on the binding of amino acyl sRNA to ribosomes, high ammonium chloride concentrations were used without addition of transfer factors or GTP. Sucrose gradient centrifugation was carried out as described by Spyrides and Lipmann,[13] and by Conway.[14] The composition of the incubation mixtures is given in the legends to the figures and tables.

Radioactive poly U was prepared as described[13] using polynucleotide phosphorylase and C^{14}-UDP with a specific activity of 6.35×10^4 cpm/mg.

Synthetic polynucleotides were purchased from Miles Chemical Co.; the ratios stated for mixed polymers are input ratios. Sodium salts of ATP and GTP were obtained from P-L Biochemicals, Inc. The trisodium salt of phosphoenolpyruvate (PEP), and pyruvate kinase were products of Boehringer und Soehne, Mannheim, Germany. C^{14}-amino acids and C^{14}-UDP were purchased from Schwarz Bio-Research, Inc. Deoxyribonuclease, 2 × crystallized, was obtained from Worthington Biochemical Corp.

Results.—Among the various tetracyclines (Table 1), chlortetracycline (CTC) was found to be slightly more active, using the poly U-directed phenylalanine incorporation as a test system; this agrees with earlier observations of Franklin.[6] In view of these results, chlortetracycline was chosen as the test substance in this study.

TABLE 1

INHIBITION OF PHENYLALANINE INCORPORATION BY TETRACYCLINES

Antibiotic	Conc. (μM)	Cpm	% Inhibition
Control		5200	—
Tetracycline	45	3109	40.2
Chlortetracycline	45	2823	45.7
Dimethyltetracycline	45	2948	43.3
Oxytetracycline	45	3302	36.5

The basic reaction mixture contained: 0.1 *M* Tris-HCl buffer pH 7.8; 0.01 *M* $MgCl_2$; 5 μg of poly U; 0.05 *M* KCl; 0.0001 *M* GTP; 0.006 *M* β-mercaptoethanol; 0.001 *M* ATP; 0.005 *M* PEP; 5 μg of PEP-kinase; 4×10^{-5} *M* of 19 amino acids, minus phenylalanine; 2×10^{-5} *M* of C^{14}-phenylalanine, specific activity 10 μC/μM; 0.25 mg of *E. coli* sRNA; and an amount of preincubated S-30 preparation containing 1.2 mg of protein. All tubes were kept at 0° and tetracyclines were added next to last before sRNA and poly U. Incubation was for 30 min at 35°. The reaction was stopped by the addition of 5% TCA, and the samples were heated for 15 min at 90° and washed on Millipore filters (0.45-μ pore size) with 5% TCA. Samples were then dried and counted in a Nuclear-Chicago gas-flow counter.

Since the discovery of the antibiotic activity of tetracyclines, the possibility that these polyhydroxylated compounds might act as chelating agents has been discussed. The various roles of the magnesium ion in the maintenance and integration of the components of the *in vitro* system made it desirable to explore first the possible effects of Mg^{++} concentration on the extent of CTC inhibition. As shown in Figure 1, no displacement of the optimum concentration of magnesium toward high concentration was found, as might have been expected if a chelating effect on this ion might have a role in the mechanism of action of the tetracycline. This result seems to exclude a gross effect; it does not seem to exclude entirely more subtle and localized effects in the reaction system.

In view of a considerable increase in the inhibition in the case of chloramphenicol[1] with added template, a study was made of the CTC effect on polymerization directed

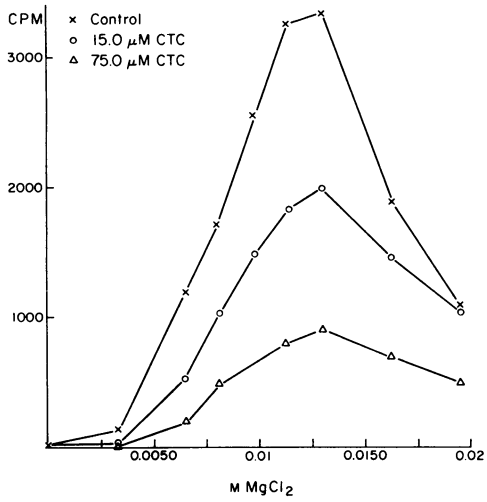

FIG. 1.—CTC inhibition of phenylalanine incorporation at various Mg^{++} concentrations. The basic reaction mixture and conditions were similar to those described in Table 1 except that the Mg^{++} concentration was varied.

by endogenous mRNA bound to the isolated ribosomes in comparison to a system to which a template had been added. As shown in Table 2, the inhibition with an already attached template was lower than if polymerization of phenylalanine was measured in the presence of added poly U. Similarly, incorporation into endogenous proteins was less affected than synthesis of protein stimulated by the addition of f2 phage RNA, as well as by turnip yellow mosaic virus (TYMV) and tobacco mosaic virus (TMV) RNA's (Table 2). Using chloramphenicol, differences were seen in inhibitory activity when different nucleotide combinations were used.[1] In that case, poly U- and poly UA-stimulated incorporation was much less inhibited than the incorporation on poly UC and poly UG as template. As shown in Table 3, the activity of CTC with various combinations shows an opposite effect, insofar as polymerization on poly U and poly UA is more affected than on poly UC, although proline incorporation on poly C is quite strongly inhibited.

Inhibition of the binding of amino acyl sRNA to the ribosomes: The results reported so far indicate, in a general manner, an interference by tetracycline on a reaction centering on the ribosome assembly process. In the hope of obtaining more precise localization, sucrose gradient centrifugation was used to study the effect of the antibiotic on the binding of sRNA as well as of the template to the ribosome. When binding of phenylalanyl sRNA to ribosomes was studied, CTC inhibited the binding by approximately 60 per cent, as shown in Figure 2. The weak attachment of sRNA in the absence of template, demonstrated by Cannon

TABLE 2

CTC INHIBITION OF PHENYLALANINE INCORPORATION STIMULATED BY ENDOGENOUS OR VIRAL RNA'S

Additions	Chlortetracycline concentrations (μM)	C^{14}-Phenylalanine Incorp. Amount (μμmoles)	% Inhibition
None	0	10.3	—
	7.5	9.1	12
	75.0	7.7	25
f2 RNA, 50 μg	0	100.6	—
	7.5	78.6	22
	75.0	43.2	67
TYMV RNA, 5 μg	0	130.2	—
	7.5	105.5	19
	75.0	65.1	50
TMV RNA, 50 μg	0	98.4	—
	7.5	78.7	20
	75.0	39.4	60

Basic reaction mixture and conditions were similar to those described in Table 1, except that poly U was either omitted or replaced by viral RNA. CTC was added before the viral RNA.

TABLE 3

INHIBITION BY CTC OF POLYMERIZATION WITH VARIOUS AMINO ACID AND TEMPLATE COMBINATIONS

Labeled amino acid	Template	% Inhibition
Lysine	Poly A	56
Proline	Poly C	75
Phenylalanine	Poly U	70
Phenylalanine	Poly UA (5:1)	50
Phenylalanine	Poly UC (1:1)	25

The components of the basic reaction mixture were essentially similar to those described in Table 1. Incubation time was 45 min. 100 μg of poly C and 5 μg of the other polymers were added to the appropriate reaction mixtures. Unlabeled amino acids were omitted from the systems containing poly A, poly C, and poly U. Polylysine was precipitated as described by Gardner *et al.*[17] and polyproline with 20% TCA. 100% incorporation represents in mμmoles: 0.5 of lysine with poly A, 0.4 of proline with poly C, and 3.4, 1.9, and 1.5 of phenylalanine with poly U, poly UA, and poly UC, respectively. Blank values were subtracted from all samples for incorporation which occurred without the addition of polymer.

et al.,[15] was not investigated. A similar experiment, carried out with C^{14}-poly U, did not show any difference of binding in the presence or absence of the antibiotic. This result, then, localizes the tetracycline interference at the binding of amino acyl sRNA to ribosomes. Our inability to show effect on the binding of poly U may not, however, definitely exclude an interference here since, in the case of chloramphenicol under similar conditions, an effect on the binding of poly UC to ribosomes could not be detected, although for various reasons[2] an interference at this phase appears most likely. A quantitative parallel between the binding, found under the conditions of gradient centrifugation, and the antibiotic effect in the polymerization reaction may thus not be expected; this might explain the

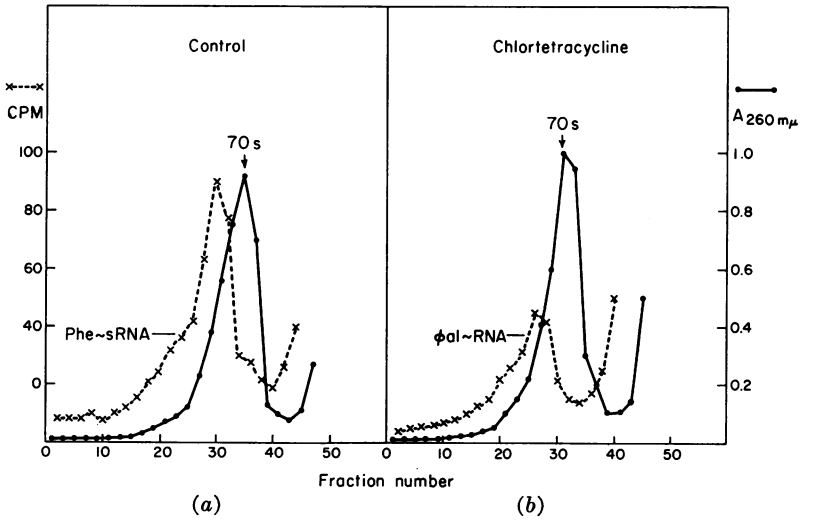

FIG. 2.—Effect of CTC on the binding of phenylalanyl-sRNA to ribosomes. Samples with 0.05 *M* Tris-HCl buffer pH 7.4, 0.012 *M* $MgCl_2$, 0.16 *M* NH_4Cl, 0.8 mg of washed ribosomes, 5 μg of poly U, and 80 μg of C^{14}-phenylalanyl-sRNA (specific activity 297 μC/μmole) in a final volume of 0.25 ml, were incubated for 10 min at 25° without (*a*) and with (*b*) 75 μM CTC. After incubation, 0.2 ml of each sample was layered over a 5–20% exponential density gradient of sucrose containing 0.05 *M* Tris-HCl buffer pH 7.4, 0.012 *M* $MgCl_2$, and 0.16 *M* NH_4Cl. The gradient onto which sample *b* was layered also included 75 μM CTC. Centrifugation was at 4° in a Spinco SW 39 rotor for 60 min at 39,000 rpm. Two-drop fractions were collected by piercing the bottom of the tubes. Absorbancy at 260 mμ was measured on even-numbered fractions (–·–) and radioactivity on odd-numbered fractions (–x–).

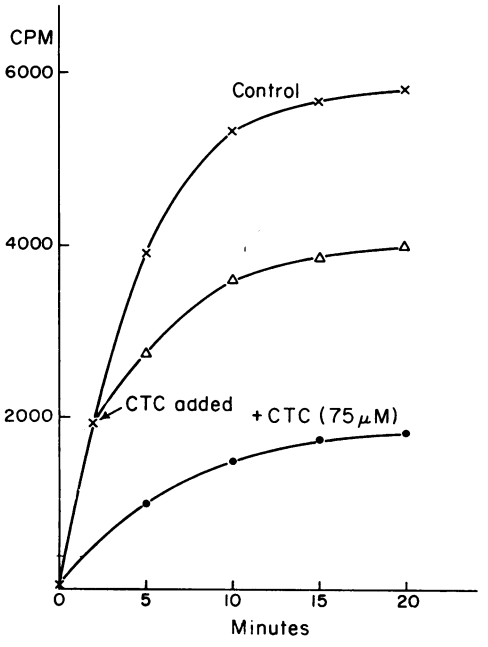

FIG. 3.—Reduction of the CTC effect by delayed addition. The complete reaction system and conditions used were similar to those described in Table 1. CTC was added to a concentration of 75 μM at zero time (-●-) and at 2 min (-△-).

incomplete inhibition of binding, although under similar conditions polymerization may be inhibited more extensively.

A localization of inhibition by the antibiotic in the assembly of components on the ribosome is further indicated by an experiment described in Figure 3, in which the effect of antibiotic was compared if addition had occurred at the beginning of the experiment or was delayed until the reaction had already started. The extent of inhibition is smaller in the latter case, as might be expected, due to the assembling of the components before the addition of inhibitor.

Summary.—It may be stated in a general manner that the tetracyclines act prior to polymerization at the stage when template and amino acyl sRNA are bound to the ribosomes. In particular, the interaction between the template-charged ribosome and amino acyl sRNA appears to be the focus of action, as a considerable inhibition of binding of amino acyl sRNA to the ribosome was demonstrated. This was the most definitely affected partial reaction in the over-all polymerization process (cf. also Suarez and Nathans[10]). It may be mentioned in this connection that a recent report by Yokota and Akiba[16] indicates the possibility of obtaining a tetracycline-resistant strain of *E. coli* that yields a resistant *in vitro* system. The resistance is located in the ribosome and is independent of the supernatant fraction used.

This work was done in Dr. Lipmann's laboratory, and I am grateful to him for many discussions and for his help in preparing the manuscript. I am also grateful for much help and advice received from Drs. T. Nakamoto and T. W. Conway.

* This work was supported in part by a grant from the National Science Foundation.

† Rockefeller Foundation fellow. Present address: Department of Physiological Chemistry, Medical Academy, Poznan, Poland.

[1] Kucan, Z., and F. Lipmann, *J. Biol. Chem.*, **239**, 516 (1964).

[2] Weisberger, A. S., and S. Wolfe, *Federation Proc.*, **23**, 976 (1964).

[3] Gale, E. F., and J. P. Folkes, *Biochem. J.*, **53**, 493 (1953).

[4] Nikolov, T. K., and A. T. Ilkov, *Abstracts*, 5th International Congress of Biochemistry, 1961, vol. 9, I-29, p. 102.

[5] Rendi, R., and S. Ochoa, *J. Biol. Chem.*, **237**, 3711 (1962).

[6] Franklin, T. J., *Biochem. J.*, **87**, 449 (1963), *ibid.*, **90**, 624 (1964).

[7] Nirenberg, M. W., and J. H. Matthaei, these PROCEEDINGS, **47**, 1580 (1961).

[8] Nathans, D., J. E. Allende, T. W. Conway, G. J. Spyrides, and F. Lipmann, in *Informational Macromolecules*, ed. H. Vogel, H. Bryson, and J. O. Lampen (New York: Academic Press, 1963), p. 349.

[9] Lipmann, F., T. P. Bennett, T. W. Conway, J. Goldstein, T. Nakamoto, and G. J. Spyrides, in *New Perspectives in Biology*, ed. M. Sela (Amsterdam: Elsevier Publishing Co., 1964), p. 69.
[10] Suarez, G., and D. Nathans, *Biochem. Biophys. Res. Commun.*, in press.
[11] Nathans, D., and F. Lipmann, these PROCEEDINGS, **47**, 497 (1961).
[12] Conway, T. W., and F. Lipmann, these PROCEEDINGS, **52**, 1462 (1964).
[13] Spyrides, G. J., and F. Lipmann, these PROCEEDINGS, **48**, 1977 (1962).
[14] Conway, T. W., these PROCEEDINGS, **51**, 1216 (1964).
[15] Cannon, M., R. Krug, and W. Gilbert, *J. Mol. Biol.*, **7**, 360 (1963).
[16] Yokota, T., and T. Akiba, *Medicine and Biology* (Japan), **64**, 39 (1962). (We are indebted to Dr. L. E. Day of Pfizer and Co. for calling our attention to this publication and for making available to us a translation from the Japanese.)
[17] Gardner, R. S., A. J. Wahba, C. Basilio, R. S. Miller, P. Lengyel, and J. F. Speyer, these PROCEEDINGS, **48**, 2087 (1962).

*AN INTERMEDIATE IN THE CONVERSION OF p-HYDROXYBENZOATE-U-C^{14} TO UBIQUINONE IN RHODOSPIRILLUM RUBRUM**

BY WILLIAM W. PARSON† AND HARRY RUDNEY‡

DEPARTMENT OF BIOCHEMISTRY, WESTERN RESERVE UNIVERSITY SCHOOL OF MEDICINE, CLEVELAND, OHIO

Communicated by Harland G. Wood, January 12, 1965

Rhodospirillum rubrum, a facultative photosynthetic anaerobe, synthesizes the benzoquinone ring of ubiquinone from p-hydroxybenzoate and p-hydroxybenzaldehyde.[1] The conversion of p-hydroxybenzoate to the ring of ubiquinone has been shown to occur in a variety of other species, including the rat.[2, 3] This process is of particular interest in *R. rubrum*, however, because little is known about the reactions by which the aromatic ring is hydroxylated under anaerobic conditions.

The carboxyl carbon atom is eliminated when p-hydroxybenzoate is converted to ubiquinone, and a methyl group from the one-carbon pool is added to the ring.[1] Assuming that the hydroxyl group of p-hydroxybenzoate is retained, three additional oxygen atoms must be introduced, two of the oxygens must receive methyl groups, and the isoprenoid side chain must be inserted. The order in which these additions occur is unknown.

A remarkable feature of the conversion of p-hydroxybenzoate and p-hydroxybenzaldehyde to ubiquinone in *R. rubrum* is its specificity. When *R. rubrum* cultures are grown for three days with p-hydroxybenzaldehyde-U-C^{14} present in the medium, essentially all of the C^{14} found in the cells is in ubiquinone and the related compound rhodoquinone.[1] (The suffix U-C^{14} indicates that the compound is uniformly labeled with C^{14}.) In an attempt to detect possible precursors of ubiquinone and rhodoquinone, short-term experiments were conducted with washed cell suspensions. With p-hydroxybenzoate-U-C^{14}, we now have observed the accumulation of a radioactive compound which appears to be an intermediate in the biosynthesis of ubiquinone. The new material has been designated provisionally as "compound X."