The Inhibition of Incorporation of Leucine into Protein of Cell-Free Systems from Rat Liver and *Escherichia coli* by Chlortetracycline

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Gale & Folkes (1953) found that chlortetracycline inhibited protein synthesis in bacterial cultures whereas nucleic acid synthesis was apparently stimulated. Nikolov & Ilkov (1961) found that it reduced the incorporation *in vivo* of methionine into the proteins of a number of tissues in the rabbit, suggesting that the drug may also interfere with protein synthesis in the animal. Although much attention has been paid in recent years to the inhibition of protein biosynthesis by puromycin, chloramphenicol and streptomycin, there appears to be little information on the mode of action of chlortetracycline in inhibiting protein biosynthesis. The present work concerns the action of this drug on the incorporation of [^{14}C]leucine into the various fractions of cell-free preparations from rat liver and *Escherichia coli*.

METHODS

Radioactive amino acid. Generally labelled L-leucine (L-[G-^{14}C]leucine; 7·7 mc/m-mole) was obtained from The Radiochemical Centre, Amersham, Bucks.

Chemicals. The sodium salt of ATP, the silver–barium salt of phosphoenolpyruvic acid and pyruvate kinase were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. The sodium salt of GTP was obtained from Pabst Laboratories, Milwaukee, Wis., U.S.A., and chlortetracycline, oxytetracycline and tetracycline hydrochlorides from Lederle Laboratories Division, Cyanamid of Great Britain Ltd., London. Chloramphenicol was provided by Dr W. H. Holms. Acid casein hydrolysate was obtained from Oxoid Division of Oxo Ltd., London.

Preparations of rat liver

Adult, white male rats of the Laboratory Wistar strain were used. The animals were starved overnight to deplete the liver of glycogen. After being killed, the animals were allowed to bleed freely and the livers were removed into an ice-cold medium (medium A) containing 0·04M-tris–HCl buffer, pH 7·7, 0·35M-sucrose, 0·01M-$MgCl_2$, 25 mM-KCl and 75 mM-NaCl. At least two livers were used to make a single preparation. The tissue was removed from the medium, blotted dry, weighed and minced with scissors. It was then homogenized in 2·5 times its weight of medium A with a Perspex pestle rotating at 1000 rev./min. in a stainless-steel tube. Nuclei, debris and mitochondria were removed by centrifuging at 15 000*g* for 15 min. at 0–1°. The middle third of the supernatant (referred to as 15S) was carefully sucked off. This was either used directly in the incorporation experiments or centrifuged for 1 hr. at 105 000*g* to sediment the microsomal pellet. The supernatant immediately above the pellet was discarded, the remainder being used to prepare the pH 5 fraction by adjusting the pH to 5·2 (glass electrode) with M-acetic acid at 0–1°. The precipitate was sedimented by centrifuging at 0° for 10 min., washed in ice-cold water and redissolved in an appropriate volume of medium A. The microsomal pellet was rinsed with ice-cold medium A and the walls of the tube were dried with tissue paper. The pellet was then resuspended in medium A by gentle homogenization with a close-fitting pestle in a Perspex tube. Ribosomes were prepared from this pellet as described by Korner (1961).

Incorporation of amino acid. A portion (1 ml.) of fraction 15S was incubated with 2 μmoles of ATP, 10 μmoles of phosphoenolpyruvate and 1 μc of [G-^{14}C]leucine (0·13 μ-mole of leucine) in a total volume of 1·5 ml. of medium having a final concentration of 8 mM-$MgCl_2$, 5·5 mM-NaCl, 1·8 mM-KCl, 3 mM-tris–HCl, pH 7·7, and 0·25M-sucrose. All additions, including chlortetracycline, were first adjusted to pH 7·7. The mixture was shaken in air at 37° for 30 min. The reaction was stopped by chilling in ice. Samples were then taken either directly for determination of radioactivity and protein content as described below or, where separation of the microsomes was required, the system was diluted with medium A containing 1·0% (w/v) of casein hydrolysate and spun at 105 000*g* for 1 hr. The pH 5 fraction was precipitated from the supernatant as described above. The microsomal pellet was rinsed with medium A and resuspended. In experiments where the pH 5 fraction was incubated without other fractions, 10 mg. of pH 5 protein was dissolved/ml. of medium A and 20 μmoles of ATP and 0·2 μc of [^{14}C]leucine were added/10 mg. of protein. This system was incubated at 37° for 10 min. The reaction was stopped by chilling in ice and the pH 5 fraction precipitated and washed as before except that the process was repeated once. Labelled transfer RNA was extracted from the pH 5 fraction by the phenol–water method of Hoagland, Stephenson, Scott, Hecht & Zamecnik (1958).

When microsomes or ribosomes were added to the pH 5 fraction, the particles were first suspended in a volume of medium A equal to the volume of fraction 15S from which they were obtained. A portion (3 ml.) of the particulate suspension (containing about 20 mg. of protein) was added to 3 ml. of solution of pH 5 fraction (containing about 15 mg. of protein) together with 6 μmoles of ATP, 6 μmoles of GTP, 30 μmoles of phosphoenolpyruvate, 100 μg. of pyruvate kinase and 3 μc of [^{14}C]leucine. [^{14}C]Leucine was not added in experiments with a prelabelled pH 5 fraction. Ionic concentrations were as described above. The time of incubation in these experiments was usually 15 min. In experiments where isolated transfer RNA was incubated

with microsomes, 1·3 mg. of prelabelled transfer RNA in 0·8 ml. of medium A was added to 12 mg. of microsomal protein suspended in 1 ml. of medium A. The final volume of 2·4 ml. contained 4 μmoles of ATP, 4 μmoles of GTP, 20 μmoles of phosphoenolpyruvate and 100 μg. of pyruvate kinase. Ionic concentrations and pH were as above. Unlabelled amino acids were not added in any incubation.

Preparations of Escherichia coli

The organism, A.T.C.C. 112299/198, was grown in 30 l. vessels at 37° with agitation and forced aeration rate of 2 l./min. in 18 l. of a medium containing (per l.): 5·4 g. of KH_2PO_4, 1·2 g. of $(NH_4)_2SO_4$, 12 g. of glucose, 0·4 g. of $MgSO_4,7H_2O$, 10 g. of Difco Bacto peptone and 1 g. of Difco yeast extract, adjusted to pH 7·1 with 5N-NaOH. The inoculum (1% of the total volume) was prepared by 'training' the organism to this medium by four successive passages in it. The cells were harvested after rapid chilling in the early log phase, the yield being about 50 g. of bacterial paste from 16 l. of medium. The cells were washed once with a solution (medium B) containing 0·01 M-tris–HCl buffer, pH 7·8, 0·01 M-magnesium acetate, 0·06 M-KCl and 6 mM-β-mercaptoethanol. Portions (4 g.) of bacterial paste were suspended in 10 ml. of medium B and treated for 2 min. in a (60 w) MSE ultrasonic oscillator. This disrupted about 70% of the cells, as judged by Gram-stained smears. Ultrasonic treatment for more than 2 min. caused a rapid loss of ribosomal activity. The product was centrifuged by the method of Nirenberg & Matthaei (1961) to ensure complete removal of intact cells from the final supernatant (fraction 30 s). The 30 s preparation was dialysed overnight at 4° against 10 vol. of medium B and then stored in small portions at −70°. It lost activity slowly. Ribosomes were prepared from this fraction by centrifuging for 2 hr. at 105 000 g. The pellet was rinsed and resuspended in medium B as described for liver particles. The pH 5 fraction was obtained in the usual way and redissolved in medium B. Transfer RNA was prepared by the method described by Hoagland *et al.* (1958).

Incorporation of amino acid. A portion (1 ml.) of fraction 30 s was incubated for 30 min. with 10 μmoles of phosphoenol pyruvate, 2 μmoles of ATP and 1 μc of [^{14}C]leucine in a total volume of 1·5 ml. The final concentrations were: 8 mM-tris, 10 mM-Mg^{2+} ions, 60 mM-K^+ ions and 5 mM-β-mercaptoethanol; pH 7·8. The activity of this system was not appreciably increased by the addition of pyruvate kinase. In experiments where ribosomes were incubated with transfer RNA, 14 mg. of ribosomal protein, suspended in 0·8 ml. of medium B, was added to 1·3 mg. of prelabelled transfer RNA dissolved in 0·6 ml. of medium B. The final volume of 2·4 ml. contained 4 μmoles of ATP, 4 μmoles of GTP, 20 μmoles of phosphoenolpyruvate and 100 μg. of pyruvate kinase. Ionic concentrations and pH were as described for the 30 s system. Time of incubation was 15 min. There were no added unlabelled amino acids in any incubation.

Measurements

Radioactivity. A slight modification of Mans & Novelli's (1961) technique was used for protein and Bollum's (1959) method for RNA. Strips of Whatman no. 3MM or no. 120 paper, 25 mm. × 40 mm., were used to support the samples. The thicker grade of paper (no. 120), for larger samples up to 0·4 ml., did not affect the counting efficiency (55%). The strips were suspended in scintillator (20 ml.) in low-potassium-glass vials. Counting errors due to variations in orientation of the strips were eliminated by using the spectrometer in the 'coincidence' position. The scintillator used contained 0·1 g. of 1,4-bis-(5-phenyloxazol-2-yl)benzene and 4 g. of 2,5-diphenyloxazole/l. of sulphur-free toluene. The instrument used was a Tricarb automatic liquid-scintillation spectrometer, model 314 EX (Packard Instrument Co. Inc.). In the early experiments the radioactivity of transfer RNA was determined after extraction with water-saturated phenol as described by Hoagland *et al.* (1958). Later, a more convenient method was developed. Duplicate samples of a solution of the pH 5 fraction were applied to the filter-paper strips. One series was washed (hot-washed) as described for proteins by Mans & Novelli (1961) to remove the RNA. The other series was washed (cold-washed) by the method described for nucleic acids by Bollum (1959), in which the RNA is left on the paper with the protein. The difference in counts/min., cold-washed − hot-washed, was assumed to be equal to the counts/min. incorporated into the transfer RNA. This technique gave specific activities for transfer RNA that were not significantly different from those obtained by the water-saturated-phenol method. RNA extracted with boiling 10% NaCl had a slightly lower specific activity.

Protein. A biuret method described by Layne (1957) was used.

Ribonucleic acid. RNA was determined by using the absorption at 260 mμ, an extinction coefficient of 34·2/mg./cm. (Korner, 1959) being assumed.

RESULTS

Effect of chlortetracycline on leucine incorporation into total protein. Chlortetracycline significantly inhibited the incorporation of leucine *in vitro* into protein by the rat-liver 15 s and *E. coli* 30 s preparations at a concentration as low as 4 μM. Over the entire range of concentrations the bacterial preparation was more sensitive to chlortetracycline than the rat-liver system. At a concentration of 400 μM there was 89% inhibition of incorporation into *E. coli* 30 s protein preparation and 72% inhibition of incorporation into liver 15 s protein preparation (Fig. 1).

Effect of chlortetracycline on leucine incorporation into transfer RNA. Table 1 shows that, in either system, chlortetracycline did not affect the attachment of leucine to the isolated transfer RNA of the pH 5 fraction. The rat-liver RNA apparently accepted leucine more readily than *E. coli* RNA, perhaps because the rat-liver preparation was less saturated with unlabelled leucine than the bacterial transfer RNA.

Effects of chlortetracycline on leucine incorporation into transfer RNA and particles. Table 2 indicates that, in the rat-liver 15 s preparation, chlortetracycline inhibited the incorporation of the amino acid into the microsomal protein, and there was also an increased labelling of the transfer RNA. In the *E. coli* 30 s system, however, although the drug

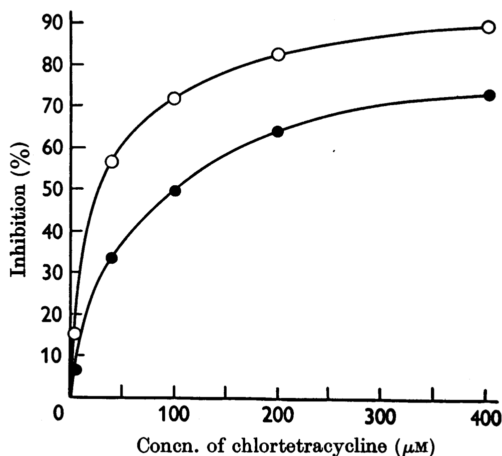

Fig. 1. Effect of chlortetracycline on incorporation of [14C]leucine into proteins of *E. coli* 30s (○) and rat-liver 15s (●) preparations. Experimental details are described in the Methods section.

Table 1. *Effect of chlortetracycline on incorporation of* [14C]*leucine into transfer RNA of* pH 5 *fractions of rat liver and* Escherichia coli

Isolated pH 5 fractions from rat liver and *E. coli* were incubated for 10 min. as described in the Methods section.

	Radioactivity incorporated (counts/min./mg. of RNA)	
	Rat liver	*E. coli*
Control	21 000	1370
Chlortetracycline added (400 μM)	22 100	1430
ATP omitted	680	56

Table 2. *Effect of chlortetracycline on incorporation of* [14C]*leucine into transfer RNA and particles of rat-liver* 15s *and* Escherichia coli 30s *preparations*

Rat-liver 15s and *E. coli* 30s preparations were incubated with additions for 30 min. as described in the Methods section.

	Radioactivity incorporated (counts/min./mg.)	
Rat-liver 15s preparation	Transfer RNA	Microsomal protein
Control	1160	252
Chlortetracycline added (400 μM)	2140	33
ATP and phosphoenolpyruvate omitted	15	8
E. coli 30s preparation	Transfer RNA	Ribosomal protein
Control	1260	320
Chlortetracycline added (400 μM)	1160	54
ATP and phosphoenolpyruvate omitted	21	22

Table 3. *Effect of chlortetracycline on* [14C]*leucine incorporation into transfer RNA and protein*

Rat-liver pH 5 fraction was incubated with preparations of rat-liver microsomes or ribosomes for 15 min. as described in the Methods section.

	Radioactivity incorporated (counts/min./mg.)	
pH 5 fraction and microsomes	Transfer RNA	Microsomal protein
Control	4380	904
Chlortetracycline added (400 μM)	7850	390
ATP, GTP, phosphoenolpyruvate and pyruvate kinase omitted	0	6
pH 5 fraction and ribosomes	Transfer RNA	Ribosomal protein
Control	2940	410
Chlortetracycline added (400 μM)	6350	210
ATP, GTP, phosphoenolpyruvate and pyruvate kinase omitted	1	4

strongly inhibited the labelling of the ribosomal protein there was no increased labelling of the transfer RNA. In further experiments a pH 5 fraction from rat liver was incubated with preparations of rat-liver microsomes or ribosomes and the effect of chlortetracycline tested. In both systems the drug inhibited the incorporation of leucine into the particulate protein and increased the labelling of the transfer RNA (Table 3). The results suggested that chlortetracycline interferes with the transfer of the amino acid from the transfer RNA to the ribonucleoprotein particle and in the next experiment this was tested directly.

Effects of chlortetracycline and other antibiotics on the transfer of leucine from transfer RNA to microsomal and ribosomal protein. The pH 5 fraction from rat liver was prelabelled with [14C]leucine as described by Hoagland *et al.* (1958). The total radioactivity present in the transfer RNA was determined. The pH 5 preparation was then added to a rat-liver microsomal preparation with the additions as described in the Methods section. After incubation for 15 min. the pH 5 fraction and particles were isolated again and their radioactivity was measured. The results in Table 4 show that chlortetracycline prevented much of the leucine of the transfer RNA being transferred to the microsomal protein. The drug-induced reduction was 3370 total counts/min. transferred to the microsomal protein, whereas the additional counts/min. retained on the transfer RNA amounted to only 1210. However, in experiments where transfer RNA labelled with [14C]leucine was extracted from the pH 5 fraction and then added to a microsomal preparation with the necessary additions, the

Table 4. *Effect of chlortetracycline on transfer of* [^{14}C]*leucine from prelabelled* pH 5 *fraction to microsomal protein in rat-liver preparations*

Prelabelled rat-liver pH 5 fraction was incubated with rat-liver microsomes for 15 min. as described in the Methods section.

	Total radioactivity (counts/min.)		
	Transfer RNA at start	Transfer RNA at end	Microsomal protein
Control	—	3190	5900
Chlortetracycline added (400 μM)	16 200	4400	2530
ATP, GTP, phosphoenolpyruvate and pyruvate kinase omitted	—	1570	88

Table 5. *Effect of chlortetracycline on transfer of* [^{14}C]*leucine from isolated transfer RNA to microsomal and ribosomal protein in rat-liver and* Escherichia coli *preparations*

Isolated prelabelled transfer RNA was incubated for 15 min. with rat-liver microsomes or *E. coli* ribosomes as described in the Methods section.

		Total radioactivity (counts/min.)		
		Transfer RNA at start	Transfer RNA at end	Microsomal protein
Rat liver	Control	—	9 090	2840
	Chlortetracycline added (400 μM)	15 600	11 200	450
			Transfer RNA	Ribosomal protein
E. coli	Control	—	10 140	2370
	Chlortetracycline added (400 μM)	15 900	13 100	239

Table 6. *Inhibition of transfer of* [^{14}C]*leucine from transfer RNA to microsomal and ribosomal protein of rat-liver and* Escherichia coli *preparations by various antibiotics*

Prelabelled transfer RNA was incubated with rat-liver microsomes or *E. coli* ribosomes for 15 min. as described in the Methods section.

	Inhibition of transfer (%)	
Antibiotic (400 μM)	Rat liver	*E. coli*
Chlortetracycline	78	93
Oxytetracycline	27	86
Tetracycline	28	90
Chloramphenicol	0	58

reduction in total counts transferred to the microsomal protein approximately equalled the additional counts retained on the transfer RNA (Table 5). Similarly, in corresponding *E. coli* preparations, chlortetracycline inhibited the transfer of leucine from transfer RNA to the ribosomal protein (Table 5). The effects of two other tetracyclines, oxytetracycline and tetracycline, and also chloramphenicol, on the amino acid transfer were studied in rat-liver and *E. coli* preparations. Oxytetracycline and tetracycline were more inhibitory in the *E. coli* than in the rat-liver system, and chloramphenicol, although moderately inhibitory to the bacterial transfer reaction, was completely without effect on the liver preparation (Table 6).

Effect of concentration of Mg^{2+} *ions on the inhibitory action.* Since chlortetracycline can act as a chelating agent (Hahn, 1958), it was possible that it inhibited the incorporation of leucine into protein by reducing the concentration of Mg^{2+} ions. However, 400 μM-chlortetracycline could remove a maximum of only 0·4 mM-Mg^{2+} ions, a very small proportion of the optimum concentrations. In fact, the addition of up to 1·2 mM-Mg^{2+} ions in excess did not release the inhibitory effect of the drug in either preparations 15S or 30S.

DISCUSSION

Chlortetracycline has been found to reduce the incorporation of [^{14}C]leucine into the ribosomal protein of cell-free systems from rat liver and *Escherichia coli*. The drug does not interfere with attachment of the amino acid to the transfer RNA in either animal or bacterial preparations but prevents the subsequent transfer of leucine to the ribosomal protein. Oxytetracycline and tetracycline appear to act in a similar way, thus confirming Hahn's prediction that the tetracyclines would inhibit protein biosynthesis by interfering with some later stage in the process (Hahn, 1958). The experiments reported, especially those in Table 5, indicate that chlortetracycline largely prevents the removal of amino acids from amino-acyl RNA. In contrast, puromycin causes a

deacylation of the amino–acyl complex (Nathans & Lipmann, 1961). In the crude rat-liver 15s preparations, however, the retention of counts on the transfer RNA resulting from inhibition by chlortetracycline is less striking and in the analogous *E. coli* preparations there is no increase in the radioactivity of the transfer RNA over the control values. The enzymic deacylation of the transfer RNA–amino acid complex described by Nathans & Lipmann (1961) may be more rapid in the cruder preparations. It is not clear at present whether chlortetracycline prevents part or all of the transfer RNA from becoming attached to the ribosomal RNA since Bloemendal & Bosch (1962) have shown that this reaction is not dependent on amino acid transfer.

It appears that the rat-liver preparations may be less sensitive to the tetracyclines than those from *E. coli*. In this context, Rendi & Ochoa (1961) have reported briefly that oxytetracycline inhibited the transfer of amino acid from transfer RNA to the ribosomal protein of a bacterial preparation but was without effect on the same reaction in a corresponding rat-liver system. Since 400 μM-oxytetracycline is only 27% inhibitory in the rat-liver system described here, as compared with 86% inhibitory action in the bacterial preparation, it may be that the drug concentration used by Rendi & Ochoa (1961) was too low for an inhibitory effect to be observed in their rat-liver system. The reason for the apparent difference in sensitivity to the tetracyclines of the animal and bacterial systems is not clear.

SUMMARY

1. Chlortetracycline has been shown to reduce the incorporation of [^{14}C]leucine into the ribosomal protein of both rat-liver and *Escherichia coli* cell-free preparations *in vitro*.

2. In neither the animal nor bacterial system did the drug interfere with the attachment of the amino acid to the transfer RNA.

3. In both preparations the drug prevented the amino acid of the transfer RNA–amino acid complex from transferring to the ribosomal protein. In highly purified systems the amino acid not transferred to the ribosome was largely retained on the transfer RNA.

4. Oxytetracycline and tetracycline also inhibited the transfer of amino acid to the ribosomal protein in both animal and bacterial systems. The rat-liver preparations were apparently less sensitive to the tetracyclines than those from *E. coli*. Chloramphenicol, inhibitory to the *E. coli* system, was without effect on the rat-liver system.

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Adenosine Triphosphate–Creatine Phosphotransferase from Ox Brain: Purification and Isolation

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Adenosine triphosphate–creatine phosphotransferase (EC 2.7.3.2) plays an important part in cerebral metabolism by maintaining concentrations of ATP under resting conditions and, after activity such as that after electrical stimulation, restoring these concentrations at the expense of the phosphocreatine of the tissue (McIlwain, 1959). Although the enzyme from skeletal muscle has