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The Effect of Chlortetracycline on the Transfer of Leucine and 'Transfer' Ribonucleic Acid to Rat-Liver Ribosomes *in vitro*

BY T. J. FRANKLIN
Imperial Chemical Industries Ltd., Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire

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Chlortetracycline prevents the passage of leucine from 'transfer' RNA to ribosomal protein in cell-free preparations from *Escherichia coli* and rat liver; the leucine is largely retained on the 'transfer' RNA (Franklin, 1963). Since attachment of 'transfer' RNA to ribosomal RNA may occur independently of amino acid incorporation into protein (Bloemendal & Bosch, 1962), it was decided to determine whether chlortetracycline prevents attachment of the amino acyl-'transfer' RNA to the ribosome or whether it prevents the subsequent condensation of amino acids into polypeptides. The present paper describes experiments with rat-liver preparations.

METHODS

Radioactive chemicals. Generally labelled L-leucine (L-[G-^{14}C]leucine) (24·8 mc/m-mole) and [6-^{14}C]orotic acid (11·6 mc/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks.

Chemicals. The sodium salt of ATP, the silver–barium salt of phosphoenolpyruvic acid, pyruvate kinase and pancreatic ribonuclease 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 hydrochloride from Lederle Laboratories Division, Cyanamid of Great Britain Ltd., London. Sodium dodecyl sulphate was obtained from L. Light and Co., Colnbrook, Bucks.

Preparation of 'transfer' ribonucleic acid labelled with [^{14}C]uracil and [^{14}C]cytosine. Each of four male adult white rats of the Laboratory Wistar strain received [^{14}C]orotic acid (0·43 μmole; 5 μc) dissolved in 0·9% sodium chloride solution (0·25 ml.) by intraperitoneal injection. The animals were killed 20 hr. later and the livers removed into an ice-cold medium (medium A) containing (final concentrations) tris–HCl buffer, pH 7·7 (40 mM), sucrose (0·35 M), $MgCl_2$ (10 mM), KCl (25 mM) and NaCl (75 mM). The pH 5 fraction (Franklin, 1963) was dissolved in water, and after the addition of sodium dodecyl sulphate (2 mg./ml.) the RNA was extracted by the method of Nirenberg & Matthaei (1961). Low-molecular-weight contaminants were removed by passage through Sephadex G-50 (Pharmacia, Uppsala, Sweden) (Bosch, Wende, Sluyser & Bloemendal, 1961). The specific radioactivity of the purified RNA was usually about 17000 counts/min./mg.

Preparation of 'transfer' ribonucleic acid labelled with [^{14}C]leucine. Rat-liver pH 5 preparations were labelled with [^{14}C]leucine (Franklin, 1963). The RNA was extracted and purified on Sephadex G-50 as described above except that the column was eluted with 1 mM-acetic acid instead of water.

Preparation of ribosomal ribonucleic acid. Ribosomes isolated after incubation as described below were resuspended in medium A and treated in the following different ways: (*a*) RNA was extracted by the method of Hoagland, Stephenson, Scott, Hecht & Zamecnik (1958) (single extraction with phenol); (*b*) RNA was extracted by the method of Nirenberg & Matthaei (1961) except that the preparation was not dialysed (triple extraction with phenol); (*c*) the ribosomes were twice extracted with 10% (w/v) sodium chloride at 100° after the washing procedure of Hoagland *et al.* (1958). The RNA was twice precipitated with ethanol.

Preparation of microsomes and ribosomes. Microsomes were prepared as described by Franklin (1963). To prepare ribosomes, the supernatant obtained after centrifuging the liver homogenate at 15000*g* for 15 min. was treated with $\frac{1}{9}$ vol. of 5% (w/v) sodium deoxycholate in 0·2 M-glycyl-glycine–NaOH buffer, pH 8·2, and centrifuged at 105000*g* for 90 min. The ribosomal pellet was resuspended in medium A (11 ml.) and centrifuged at 105000*g* for 90 min. Finally microsomes and ribosomes from 25 g. of liver were resuspended in medium A (10 ml.). In ribosomal preparations a small portion of the 15000*g* supernatant, not treated with sodium deoxycholate, was centrifuged at 105000*g* for 90 min. to obtain the final supernatant (105000*g* supernatant).

Conditions of incubation. A portion (2 ml.) of the particulate suspension (containing about 6 mg. of RNA)

Table 1. *Effect of chlortetracycline on the binding of 'transfer' ribonucleic acid labelled with* [^{14}C]*uracil and* [^{14}C]*cytosine to microsomes and ribosomes*

'Transfer' RNA was incubated with microsomes or ribosomes for 15 min. as described in the Methods section. In Expt. 2 microsomes were converted into ribosomes after the incubation but before the determination of radioactivity.

		Total radioactivity (counts/min.)	
		'Transfer' RNA at start	Ribosome at end
Expt. 1	Microsomes:		
	Control	8800	341
	Chlortetracycline (0·4 mM) added		348
	ATP, GTP, phosphoenolpyruvate, pyruvate kinase and 105000*g* supernatant omitted		200
Expt. 2	Microsomes:		
	Control	6100	1500
	Chlortetracycline (0·4 mM) added		1600
	ATP, GTP, phosphoenolpyruvate, pyruvate kinase and 105000*g* supernatant omitted		850
Expt. 3	Ribosomes:		
	Control	4700	1200
	Chlortetracycline (0·4 mM) added		1100
	ATP, GTP, phosphoenolpyruvate, pyruvate kinase and 105000*g* supernatant omitted		520

was incubated with 30 μmoles of phosphoenolpyruvate, 6 μmoles of ATP, 1 μmole of GTP, 100 μg. of pyruvate kinase, 0·3 mg. of 'transfer' RNA or [^{14}C]leucine (for the amount see the Results section) and 0·1 ml. of 105000*g* supernatant (containing about 2 mg. of protein) in a total volume of 4·3 ml. of medium containing (final concentrations) $MgCl_2$ (8 mM), NaCl (5·5 mM), KCl (1·8 mM), tris–HCl buffer, pH 7·7 (3 mM), and sucrose (0·25 M). All additions, including chlortetracycline (0·4 mM), were first adjusted to pH 7·7. No unlabelled amino acids were added. The mixture was shaken in air at 37° for 15 min. The reaction was stopped by chilling in ice; the mixture was diluted with ice-cold medium A and centrifuged at 105000*g* for 90 min. The pellets were resuspended in medium A (10 ml.) and in some experiments microsomes were converted into ribosomes by adding 5% (w/v) sodium deoxycholate (1·1 ml.). The suspensions were centrifuged for 90 min. at 105000*g* and resuspended in a suitable volume of medium A. The measurement of radioactivity in the intact particles or in the extracted ribosomal RNA was as described by Franklin (1963).

Sucrose-density-gradient analysis of ribosomal ribonucleic acid. Ribosomal RNA (600 mg.) prepared by method (*b*) was dissolved in 5% (w/v) sucrose in 40 mM-tris–HCl buffer, pH 7·7 (0·2 ml.), and layered on top of a linear density gradient of 5–20% (w/v) sucrose in 40 mM-tris–HCl buffer, pH 7·7, the total volume being 4·7 ml. The gradient was prepared by using the device described by Britten & Roberts (1960). The tubes were centrifuged at 20000 rev./min. for 16 hr. at 4° in the SW 39 rotor of the Spinco model L centrifuge. At the end of the run the tubes were punctured with a hypodermic needle sealed into the tube with silicone grease. Fractions (22 drops) were collected directly on strips of Whatman no. 120 paper (25 mm. × 40 mm.) for radioactivity measurements (Franklin, 1963). From a duplicate tube fractions (11 drops) were collected for measurement of the extinction at 260 mμ ($E_{260\,m\mu}$) after dilution with water (1 ml.).

Determination of protein. The method described by Lowry, Rosebrough, Farr & Randall 1951) was used.

Determination of ribonucleic acid. With $E_{260\,m\mu}$, an extinction coefficient of 34·2/mg./cm. (Korner, 1959) was assumed. All samples were heated with 0·5 N-perchloric acid before measurement of the extinction.

RESULTS

Effect of chlortetracycline on the binding of 'transfer' ribonucleic acid labelled with [^{14}C]*uracil and* [^{14}C]*cytosine to microsomes and ribosomes.* Three types of experiment were performed, with incubation of the 'transfer' RNA with: (*a*) microsomes; (*b*) microsomes with conversion into ribosomes after incubation (Bosch, Bloemendal & Sluyser, 1960); (*c*) ribosomes. After incubation the radioactivity of the particles was measured. Chlortetracycline (0·4 mM), which strongly inhibits the transfer of amino acid from 'transfer' RNA to particulate protein (Franklin, 1963), had no marked effect on the attachment of 'transfer' RNA to microsomes or ribosomes (Table 1). A high level of binding of radioactivity to the particles occurred in the absence of added ATP, GTP, phosphoenolpyruvate and 105000*g* supernatant. Other workers have encountered this effect (Hoagland & Comly, 1960; Bosch, Bloemendal, Sluyser & Pouwels, 1961), and with our preparations it is very difficult to decrease.

Effect of chlortetracycline on the transfer of leucine to ribosomal ribonucleic acid. Moldave (1960) reported an energy-dependent attachment of leucine to microsomal RNA in cell-free systems from rat liver. The effect of chlortetracycline on the

transfer of leucine to ribosomal RNA was tested. The radioactivity of ribosomal RNA after incubation of ribosomes with either 'transfer' RNA labelled with [^{14}C]leucine or free [^{14}C]leucine was determined by four different methods: (*a*) By determination of the difference in radioactivity of duplicate samples of the ribosomal suspension applied to filter-paper strips washed either in cold or in cold and then hot trichloroacetic acid (Franklin, 1963). (*b*) By direct measurement of the radioactivity of ribosomal-RNA samples extracted from the ribosomes by the three different techniques described in the Methods section. Chlortetracycline strongly inhibited the transfer of leucine from 'transfer' RNA to ribosomal RNA (Table 2); in this experiment the radioactivity of the RNA was determined by the difference in counts/min. between cold-washed and cold-washed–hot-washed ribosomes. The transfer was dependent on the presence of phosphoenolpyruvate and the 105000*g* supernatant. The specific radioactivity of the ribosomal leucine extractable with hot trichloroacetic acid, if it is related to ribosomal RNA, showed striking differences depending on the method of isolation of RNA (Table 3). The specific radioactivity determined by the difference in counts/min. between cold-washed and cold-washed–hot-washed ribosomes, expressed as counts/min./mg. of ribosomal RNA extracted by 0·5N-perchloric acid at 70°, was about five times the value for RNA samples isolated by triple extraction with phenol (Nirenberg & Matthaei, 1961) and which contained 2% or less of protein. The radioactivity of these preparations was also greatly decreased by the presence of chlortetracycline in the incubation mixture and by the absence of phosphoenolpyruvate and 105000*g* supernatant (Fig. 1). RNA prepared by the single-phenol-extraction procedure of Hoagland *et al.* (1958) contained 30–40% of protein and the specific radioactivity of these preparations, which was released by hot trichloroacetic acid, was similar to that determined by direct determinations on the ribosomes. RNA extracted into boiling aqueous sodium chloride contained 4% of protein and its specific radioactivity, soluble in hot acid, was similar to that of RNA isolated by the method of Nirenberg & Matthaei (1961).

Table 2. *Effect of chlortetracycline on the transfer of leucine from 'transfer' ribonucleic acid to ribosomal protein and ribonucleic acid*

'Transfer' RNA labelled with [^{14}C]leucine (8000 counts/min.) was incubated for 15 min. with ribosomes as described in the Methods section. The difference in counts/min. between cold-washed and cold-washed–hot-washed ribosomes was assumed to be equal to counts/min. incorporated into ribosomal RNA.

	Radioactivity incorporated (counts/min./mg.)	
	Ribosomal protein	Ribosomal RNA
Control	325	133
Chlortetracycline (0·4 mM) added	23	23
105000*g* Supernatant omitted	20	13
Phosphoenolpyruvate omitted	31	23
ATP, GTP, phosphoenolpyruvate, pyruvate kinase and 105000*g* supernatant omitted	10	0

Effects of various treatments on the radioactivity of ribosomal ribonucleic acid labelled with [^{14}C]*leucine.* Ribosomal RNA was extracted by triple extraction with phenol (Nirenberg & Matthaei, 1961) from ribosomes incubated with [^{14}C]leucine (0·32 μmole; 8 μc). Table 4 indicates that treatment of the RNA with 5% (w/v) trichloroacetic acid at 90° for 30 min. removed all the radioactivity precipitable in ice-cold trichloroacetic acid. Incubation of the RNA with 0·02 part (by wt.) of pancreatic ribonuclease released 88% of the radioactivity into solution in 20 min.; the radioactivity was stable in

Table 3. *Effect of extraction procedure on the specific radioactivity of ribosomal ribonucleic acid isolated from ribosomes labelled with* [^{14}C]*leucine*

Ribosomes were incubated with [^{14}C]leucine (0·32 μmole; 8 μc) as described in the Methods section. The particles were isolated from the incubation mixture and the specific radioactivity of ribosomal RNA was determined after treatment of the ribosomes as outlined below.

	Radioactivity soluble in hot trichloroacetic acid (counts/min./mg. of RNA)	Protein in RNA (%)
Difference in counts/min./mg. between cold-washed and cold-washed–hot-washed ribosomes	930	Ribonucleoprotein particles
RNA isolated by single phenol extraction	1000	38
RNA isolated by threefold phenol extraction in the presence of 0·2% sodium dodecylsulphate	189	1·7
RNA isolated by extraction into boiling 10% sodium chloride	224	4

the absence of ribonuclease. Incubation of the RNA for 1 hr. in 40 mM-tris–hydrochloric acid buffer, pH 7·7, with or without sodium dodecyl sulphate (0·5%), was without effect on the radioactivity.

Sucrose-density-gradient analysis of ribosomal ribonucleic acid labelled with [^{14}C]*leucine.* Fig. 1 shows the distribution of $E_{260\,m\mu}$ and the radioactivity of ribosomal RNA isolated by triple extraction with phenol (Nirenberg & Matthaei, 1961) after centrifugation in a sucrose density gradient. There are two major components and one minor component with extinctions at 260 mμ. On the assumption that the peaks have sedimentation coefficients of 28 s, 18 s and 4 s (left to right; Hiatt, 1962), all the radioactivity was present in the 4 s component, which corresponds to 'transfer' RNA. The presence of chlortetracycline in the incubation mixture greatly decreased the radioactivity of the 4 s fraction, and omission of phosphoenolpyruvate and the 105000*g* supernatant almost abolished the radioactivity (not shown). The $E_{260\,m\mu}$ pattern was unaffected by these treatments.

Table 4. *Effect of different treatments on radioactivity of ribosomal ribonucleic acid labelled with* [^{14}C]*leucine*

RNA was isolated by triple extraction with phenol (Nirenberg & Matthaei, 1961) from ribosomes incubated with [^{14}C]leucine, as described in the Methods section.

Treatment	Radioactivity (counts/min./mg. of RNA) Before treatment	After treatment
5% Trichloroacetic acid at 90° for 30 min.	288	3
Ribonuclease at 37° for 20 min. at pH 7·7	208	25
Incubation at 37° for 1 hr. at pH 7·7	615	613
Incubation at 37° for 1 hr. in 0·5% sodium dodecyl sulphate at pH 7·7	439	420

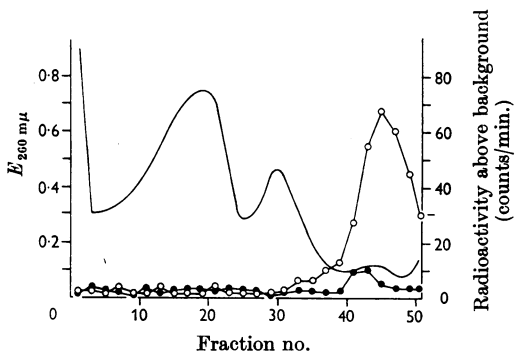

Fig. 1. Sucrose-density-gradient analysis of RNA extracted from ribosomes, labelled with [^{14}C]leucine, by a threefold extraction with phenol in the presence of 0·2% sodium dodecyl sulphate. ——, Extinction of the fractions; ○, radioactivity of ribosomal RNA from complete system not containing chlortetracycline; ●, radioactivity of ribosomal RNA from complete system also containing chlortetracycline (0·4 mM). Details of the incubation mixtures, the composition of the gradient and the centrifugation are described in the Methods section.

DISCUSSION

Gilbert (1963) has proposed a model for protein biosynthesis in *E. coli* preparations. In his view the attachment of the amino acyl-'transfer' RNA to the peptide-synthesizing site on the 50 s subunit of a 70 s ribosome, activated by 'messenger' RNA, is determined by the ability of the coded zone of the 'transfer' RNA to pair with the complementary bases of the 'messenger' RNA. The amino acyl-RNA is said to be held against the ribosomal surface by secondary hydrogen and magnesium bonds. This step, in contrast with the subsequent peptide condensation, would not require energy or enzymes. After peptide-bond formation the nascent peptide chain retains the 'transfer' RNA at the *C*-terminal end in an ester linkage. In the absence of protein biosynthesis an incoming 'transfer' RNA, charged or uncharged with an amino acid, could exchange only with a 'transfer' RNA on the ribosome not attached to a peptide chain. If this model is correct and applicable to a rat-liver system, the energy-dependent transfer of leucine to a ribosomal fraction that is soluble in hot trichloroacetic acid may represent the incorporation of the amino acid into an incomplete peptide chain bound to a 'transfer'-RNA molecule. The association of at least part of the radioactivity with the fraction corresponding to 'transfer' RNA in a sucrose-density-gradient analysis of ribosomal RNA supports this view; Gilbert (1963) observed the sedimentation of polyphenylalanyl-'transfer' RNA in the 4 s fraction of ribosomal RNA derived from *E. coli* ribosomes incubated with phenylalanine and polyuridylic acid. The insolubility of the hypothetical leucine-containing peptide in cold trichloroacetic acid could be due to the 'transfer' RNA, and treatment with hot acid, which hydrolyses the RNA, might release the small incomplete peptide into solution. The action of ribonuclease in rendering the radioactivity soluble in cold acid is in accordance with this suggestion.

Moldave (1960) observed a very small incorporation of leucine into microsomal RNA *in vitro* by measuring the radioactivity of the RNA isolated with boiling aqueous sodium chloride. The present work indicates that ribosomal-RNA preparations labelled with [^{14}C]leucine liberate more radioactivity into solution on treatment with hot acid

when they contain protein (precipitated ribonucleoprotein particle or incompletely separated RNA) than when the RNA is almost free from protein [isolated by the methods of Moldave (1960) or Nirenberg & Matthaei (1961)]. The explanation for this result is not clear, but it emphasizes the difficulties in the determination of the incorporation of an amino acid into a complex biological entity, such as the ribosome, by measuring the radioactivity of one of its components isolated by a rigorous extraction procedure.

Though chlortetracycline strongly inhibits the incorporation of leucine into a ribosomal fraction soluble in hot trichloroacetic acid, the drug has no marked effect on the binding of [^{14}C]pyrimidine-labelled 'transfer' RNA to the ribosome. The energy-dependent binding of 'transfer' RNA to the ribosome may be due to the attachment of the nucleic acid to the *C*-terminal end of the nascent peptide chain after peptide-bond formation (Gilbert, 1963). Since leucine that is not transferred to the ribosome in the presence of chlortetracycline is largely retained on the 'transfer' RNA of the pH 5 fraction (Franklin, 1963), and since the drug does not inhibit the energy-free binding of 'transfer' RNA to the ribosome, it might have been expected to inhibit the energy-dependent attachment of 'transfer' RNA to the ribosome. The high level of energy-free binding of 'transfer' RNA to the ribosome, which may have obscured an inhibitory effect of chlortetracycline on the energy-dependent transfer, may be due to the large numbers of inactive ribosomes which are present in our type of preparation (Wettstein, Staehelin & Noll, 1963) and which could involve a considerable proportion of the 'transfer'-RNA molecules in the non-specific binding described by Gilbert (1963). The very low level of energy-free binding of leucine to the ribosome is presumably due to the small proportion of the 'transfer' RNA (5% or less; Zamecnik, 1962) which is leucyl-'transfer' RNA. The apparent absence of an inhibitory effect of chlortetracycline on the energy-dependent binding of 'transfer' RNA to the ribosome is difficult to explain in terms of Gilbert's model of 'transfer' RNA–ribosome interaction. However, Bloemendal & Bosch (1962) and Takanami (1962) have observed an energy-dependent binding of 'transfer' RNA, stripped of amino acids, to ribosomal RNA; this is also inconsistent with Gilbert's view.

In conclusion, the present results and those of Franklin (1963) provide some indirect evidence that chlortetracycline may inhibit protein biosynthesis *in vitro* by preventing in some way the formation of the peptide bond. Further work, however, is necessary to prove this suggestion.

SUMMARY

1. Chlortetracycline apparently had no effect on the energy-dependent binding of 'transfer' RNA labelled with [^{14}C]uracil and [^{14}C]cytosine to microsomes or ribosomes in cell-free systems from rat liver.

2. Chlortetracycline strongly inhibited the transfer of [^{14}C]leucine to a fraction of the ribosome which was insoluble in cold trichloroacetic acid but was released by hot trichloroacetic acid.

3. The transfer of leucine to this fraction was dependent on the presence of phosphoenolpyruvate and the 105000*g* supernatant.

4. The observed radioactivity of ribosomal RNA labelled with [^{14}C]leucine varied considerably, depending on the method of extraction of RNA.

5. Sucrose-density-gradient analysis of ribosomal RNA labelled with [^{14}C]leucine indicated that all the radioactivity was in the 4s fraction.

6. The significance of these results is discussed in relation to the mode of action of chlortetracycline and current views on the 'transfer' RNA–ribosome interaction.

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