Ribonucleic Acid of Chloramphenicol-Treated *Shigella flexneri*

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SUMMARY

Resting organisms of *Shigella flexneri* serotype 3 are able to synthesize soluble and ribosomal ribonucleic acid (RNA) in the presence of chloramphenicol. The antibiotic stimulates the synthesis of soluble RNA but has no apparent effect on ribosomal RNA production. In contrast, chlortetracycline, which also suppresses formation of protein, stimulates soluble RNA synthesis and inhibits ribosomal RNA synthesis. The soluble RNA of the chloramphenicol-treated organisms possesses amino acid accepting activity comparable to that of the soluble RNA of untreated organisms. The findings indicate that chloramphenicol does not promote the synthesis of biologically inactive soluble RNA. The stimulation of soluble RNA synthesis appears to be the result, rather than the cause, of the inhibition of protein production by the antibiotic.

INTRODUCTION

Protein synthesis in bacteria is inhibited markedly by chloramphenicol; in contrast, ribonucleic acid (RNA) synthesis is not affected and may even be stimulated (Gale & Folkes, 1953; Wisseman, Smadel, Hahn & Hopps, 1954). Since RNA plays a major role in the production of protein (Chantrenne, 1961), it has been postulated that the inhibitory action of chloramphenicol is the result of the formation of non-functional RNA (Gale, 1958; Ramsey, 1958). The results of some of the studies of the RNA of chloramphenicol-treated *Escherichia coli* suggest that this nucleic acid is 'abnormal' and perhaps physiologically inactive. Neidhardt & Gros (1957) showed that the RNA produced in the presence of chloramphenicol, 'chloramphenicol-RNA', was unstable, being degraded when the treated organisms were placed in chloramphenicol-free medium. Other workers have shown that chloramphenicol-RNA was present in ribonucleoprotein particles of sizes smaller than those present in untreated organisms (Dagley & Sykes, 1959; Nomura & Watson, 1959). Horiuchi, Horiuchi & Mizuno (1959) observed that, after being washed and resuspended in drug-free medium, chloramphenicol-treated *Escherichia coli* showed a lag period before beginning to synthesize protein. The authors conclude that chloramphenicol-RNA does not participate in protein synthesis.

Evidence that chloramphenicol-RNA is similar to the RNA of untreated organisms has been reported; Pardee & Prestidge (1956) found that the base composition of both types of RNA was the same. Aronson & Spiegelman (1961) showed that chloramphenicol-RNA was converted to a stable form when chloramphenicol-treated *Escherichia coli* was resuspended in a medium which permitted rapid protein formation. They also presented data which demonstrated that organisms con-

taining chloramphenicol-RNA produced protein without any detectable nucleic acid synthesis. The authors have proposed that chloramphenicol-RNA is not abnormal but is a normal unstable intermediate of ribonucleoprotein and that chloramphenicol blocks the conversion of the intermediate to a stable form.

We have made experiments designed to characterize further the synthesis of RNA in the presence of chloramphenicol and the biological activity of some of the RNA. A previous study showed that chloramphenicol stimulated RNA production in resting *Shigella flexneri* serotype 3 (Yee, Pan & Gezon, 1962). The present paper presents evidence which indicates that the soluble RNA synthesis and not the ribosomal RNA synthesis in this organism is stimulated by chloramphenicol and that the soluble RNA can bind amino acids. A preliminary report of some of the findings was published elsewhere (Yee, Gezon & McElligott, 1962).

METHODS

Cultivation and starvation of organisms. *Shigella flexneri* serotype 3 strain B-1003 (Pan, Yee & Gezon, 1957) was used. The previously described methods for growing, starving and harvesting the organisms in phosphate buffered saline (pH 6.9) to prepare resting suspensions (Yee, Pan & Gezon, 1962) were modified as follows, mainly to accommodate the large volume of organisms required: (1) a sufficient quantity of organism was obtained by growing them in flasks containing 3.5 l. defined medium; (2) each flask was seeded with 5 ml. of an 18 hr. starter culture which had been inoculated with a loopful of growth from a defined-medium slope culture; (3) cultures were aerated on a rotary shaker; (4) the defined medium with glucose as carbon and energy source and ammonium monobasic phosphate as nitrogen source did not contain nicotinamide since this was not needed for optimum growth of the organism (Pan, Yee & Gezon, 1962).

Treatment with chloramphenicol. Defined medium containing L-aspartic acid as sole nitrogen source was used to obtain maximum RNA synthesis. The starved organisms were resuspended in the medium described before (Yee, Pan & Gezon, 1962). Equal volumes of suspension were added to each of three flasks. One flask serving as a 0 hr., or unincubated, control was centrifuged immediately in a refrigerated Sharples supercentrifuge. Chloramphenicol was added to the second flask to final concentration 2 μ g./ml.; the third flask was used as a chloramphenicol-free control. The latter two flasks were incubated at 37° on a rotary shaker for 4 hr. and the organisms then collected on a Sharples supercentrifuge and stored at -20°.

Separation of soluble and ribosomal RNA. A procedure based on the method of Tissières, Watson, Schlessinger & Hollingworth (1959) was used. The organisms were disrupted by grinding in a mortar with alumina A301 and extracted with 0.01 M-Tris(hydroxymethyl)aminomethane buffer (pH 7.4) containing 0.01 M-magnesium acetate to minimize degradation of ribosomal particles. Alumina, whole organisms and cell debris were removed by centrifugation at 6000g at 0°. The resulting crude extract was centrifuged at 100,000g for 120 min. to sediment ribosomal RNA. The upper third of the supernatant fluid was removed for analysis of soluble RNA; RNA and protein were precipitated with cold 5% (w/v) trichloroacetic acid. The RNA was extracted from the precipitates with hot trichloroacetic acid by the method of Gale & Folkes (1953) and measured by the orcinol reaction (Schneider, 1957) with adenosine-5'-monophosphate as standard.

Isolation of soluble RNA. Soluble RNA was isolated by phenol treatment of supernatant fluids of crude extracts obtained after centrifugation at 100,000g (Tissières, 1959). Polysaccharide was removed from the RNA preparations by treatment with 2-methoxyethanol (Kirby, 1956). The RNA was stored at -20° .

Amino acid accepting activity. The total amino acid accepting activity of the soluble RNA of untreated and of chloramphenicol-treated organisms was measured by the assay system of Berg & Ofengand (1958) using a ^{14}C algal protein hydrolysate as a source of labelled amino acids. After incubation the soluble RNA was re-isolated (Ofengand, Dieckmann & Berg, 1961) and its radioactivity measured in a Nuclear-Chicago counter equipped with a Micromil window. The ability of the soluble RNA preparations to accept individual amino acids was determined by the method of Brown (1960). The amino acid + RNA complexes were removed from the reaction system by charcoal (Norite A) adsorption and re-isolated by phenol treatment of the charcoal. The amino acids were released from the soluble RNA at pH 10 and separated by two-dimensional paper chromatography. Butanol + water + glacial acetic acid (60 + 25 + 15, by vol.), and water-saturated phenol containing 0.002% 8-hydroxyquinoline in an atmosphere of ammonia, were used as solvents. Development in each solvent was for 24 hr. The amino acid spots were located with ninhydrin, cut out, eluted with 10% (v/v) isopropanol in water and the radioactivity of the solutions measured.

RESULTS

Effect of chloramphenicol on the synthesis of soluble and ribosomal RNA. Experiments were made to determine whether soluble and/or ribosomal RNA synthesis was stimulated by chloramphenicol. Initially attempts were made to extract soluble RNA from dried organisms of untreated and chloramphenicol-treated *Shigella flexneri* with hot sodium dodecylsulphate by the method of Ofengand *et al.* (1961). However most of the RNA of the organisms was removed, indicating that ribosomal RNA also was present in the preparations. Fractionation of lysates of spheroplasts also was unsuccessful. The bulk of the cellular RNA was found in the supernatant fluids of the lysates after centrifugation at 100,000g.

A procedure which used alumina grinding to disrupt the organisms was found to be suitable. The organisms were able to synthesize both soluble and ribosomal RNA in the presence of chloramphenicol (Table 1). This finding is consistent with those reported for *Escherichia coli* (Nomura & Watson, 1959; Aronson & Spiegelman, 1961). In addition, soluble RNA production in resting *Shigella flexneri* was stimulated by chloramphenicol whereas ribosomal RNA production appeared to be unaffected. As a result, the chloramphenicol-treated organisms possessed low ribosomal RNA: soluble RNA ratios. It should be pointed out that the degree of RNA synthesis varied with different preparations; however, the ratios were relatively constant and reproducible.

The cell-wall and membrane fractions of the disrupted organisms were also examined. The RNA content of the fractions was less than 0.5% of the total cellular RNA and no detectable increase occurred during the incubation of untreated or chloramphenicol-treated organisms.

Effect of chlortetracycline on RNA synthesis. Chlortetracycline also inhibits protein synthesis (Gale & Folkes, 1953). The action of this antibiotic on RNA synthesis

was compared to that of chloramphenicol. The results indicated that the observed effect of chloramphenicol on RNA synthesis was not a non-specific one, i.e. antibiotics which inhibited protein synthesis might not be similar in their action on

Table 1. *Effect of chloramphenicol on RNA synthesis by resting Shigella flexneri serotype 3*

Resting organisms were prepared by washing and starving freshly harvested organisms in phosphate buffered saline (pH 6.9). The organisms were harvested by centrifugation and resuspended in a solution of KH_2PO_4 , Na_2HPO_4 and NaCl ; the organism concentration was adjusted so that the suspension gave 10% light transmittance at 425 m μ with a light path of 13 mm. in a Coleman model 6B spectrophotometer. Two volumes of the suspension were mixed with one volume of a solution containing L-aspartic acid as sole nitrogen source, D-glucose and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. The final concentration of all compounds was the same as that in the defined medium used for growth of the organisms. This suspension containing c. 2.5×10^8 resting organisms/ml. was incubated on rotary shaker at 37°.

Organism preparation	Cell fraction	Expt. 1			Expt. 2		
		RNA content*	Net increase	Ribo-somal:soluble	RNA content	Net increase	Ribo-somal:soluble
0 hr. (before incubation)	Ribosomal†	104	—	2.9:1	75	—	2.4:1
	Soluble	36	—		31	—	
	Total	140	—		106	—	
Incubated 4 hr. without chloramphenicol	Ribosomal	127	23	3.2:1	106	31	3.2:1
	Soluble	39	3		33	2	
	Total	166	26		139	33	
Incubated 4 hr. with 2 μg . chloramphenicol/ml.	Ribosomal	119	15	1.8:1	121	46	2.0:1
	Soluble	65	29		60	31	
	Total	184	44		181	77	

* Expressed as μmole RNA nucleotide/ml. organism suspension.

† Calculated value (total minus soluble). Total and soluble RNA were determined by chemical analysis.

Table 2. *Effect of antibiotics on protein and RNA synthesis by Shigella flexneri serotype 3*

Antibiotic concentration ($\mu\text{g./ml.}$)	Change in amount of synthesis (%)	
	Protein	RNA
Chloramphenicol		
0.5	−24*	+18*
1	−41	+36
2	−76	+68
Chlortetracycline		
0.1	−15	+92
0.25	−71	+54
0.5	−94	+12

* −, Decrease; +, increase.

RNA synthesis. The effect of chlortetracycline on protein and RNA synthesis is shown in Table 2; the action of chloramphenicol is shown for comparison. Concentrations of chlortetracycline less than the minimum required for complete inhibition of protein synthesis and cell multiplication stimulated RNA synthesis. As the chlortetracycline concentration was increased the degree of stimulation

decreased, contrary to the finding with chloramphenicol. Also, chlortetracycline not only stimulated soluble RNA synthesis but also decreased the production of ribosomal RNA (Table 3).

Table 3. *Effect of chlortetracycline on RNA synthesis by resting Shigella flexneri serotype 3*

Organism preparation	Cell fraction	Net increase, $m\mu$ mole RNA nucleotide/ml. suspension
Incubated 4 hr. without antibiotic	Ribosomal	105
	Soluble	17
	Total	122
		(3.8:1)*
Incubated 4 hr. with antibiotic	Ribosomal	55
	Soluble	50
	Total	105
		(1.8:1)

* Numbers in parentheses represent ratio of ribosomal RNA to soluble RNA.

Organism suspension and incubation same as in Table 1. Chlortetracycline concentration, 0.5 μ g./ml.

Table 4. *Amino acid accepting ability of soluble RNA of untreated and chloramphenicol-treated Shigella flexneri serotype 3*

Source of soluble RNA	RNA preparations		
	I	II	III
Untreated organisms	1877*	2400	1833
Chloramphenicol-treated organisms	2163	2300	1900

* Counts/min./ μ mole RNA nucleotide.

Table 5. *Amino acid accepting ability*

Amino acid	Source of soluble RNA	
	Untreated organisms	Chloramphenicol-treated organisms
Leucine	41.2*	39.0
Isoleucine		
Phenylalanine		
Tyrosine	19.3	20.6
Arginine	8.0	8.6
Valine	3.9	5.0
Glutamic	5.0	5.8
Aspartic	4.7	4.6
Histidine	3.9	3.5
Alanine	4.0	4.4
Glycine	2.5	2.0
Serine	2.0	1.8
Proline	2.0	1.9
Threonine	1.8	1.5
Lysine	1.7	1.5

* Percentage of total count.

Amino acid accepting ability. Soluble RNA mediates protein synthesis by binding amino acids and transporting them to the ribosomes where peptide bonding occurs (Chantrenne, 1961). The production of non-functional RNA may cause diminution of protein synthesis. Eaton & Caffrey (1961) reported that the soluble RNA formed by *Escherichia coli* in the presence of dihydrostreptomycin was unable to accept amino acids. It seemed feasible to test the soluble RNA produced by chloramphenicol-treated *Shigella flexneri* (soluble chloramphenicol-RNA). As a working hypothesis it was assumed that the stimulation of soluble RNA synthesis by chloramphenicol might be an indication that the nucleic acid was 'abnormal' and physiologically inactive. As shown in Table 4, the soluble chloramphenicol-RNA had a total amino acid accepting ability which was comparable to that of the soluble RNA of untreated organisms. Similar results were obtained when the accepting ability for individual amino acids was determined (Table 5). These findings indicate that chloramphenicol does not induce the formation of nonfunctional soluble RNA in *S. flexneri* serotype 3.

DISCUSSION

The conclusion that chloramphenicol stimulates soluble RNA synthesis is based on the observation that resting chloramphenicol-treated *Shigella flexneri* had a higher soluble RNA content than untreated resting organisms. Alternative explanations which might be given for this finding can be excluded. The resting suspensions were prepared by washing and starving the organisms in phosphate buffer saline. This solution was used in an earlier study in which the stimulation of total RNA synthesis in *S. flexneri* by chloramphenicol was shown (Yee *et al.* 1962*a*). The starvation of the organisms in a magnesium-free medium may have resulted in a lower ribosomal RNA:soluble RNA ratio, indicating that degradation of ribosomes may occur. This did not affect the results. Chloramphenicol-treated organisms which had been washed and starved in a magnesium-containing solution to maintain the integrity of the ribosomes also had a higher soluble RNA content than untreated organisms (Yee *et al.* 1962*b*).

The use of alumina grinding to disrupt the organisms may have introduced an error. Aronson & Spiegelman (1961) reported that the use of this method to disrupt *Escherichia coli* resulted in the release of some of the ribosomal chloramphenicol-RNA into the soluble fraction. In addition, the observed increase in the soluble RNA of chloramphenicol-treated *Shigella flexneri* may be attributed to degradation of the ribosomal RNA in the organisms as a result of exposure to the antibiotic, or to the inability of the treated organisms to assemble newly formed ribosomal RNA subunits. However, the amino acid accepting activities of soluble chloramphenicol-RNA and soluble RNA of untreated organisms are similar. The above alternative explanations would require the unlikely assumption that functional soluble RNA is a degradation product or a precursor of ribosomal RNA. Lacks & Gros (1959) were unable to show any conversion of soluble RNA to ribosomal RNA. On the basis of this finding and the difference in the base composition of the two types of RNA, they concluded that soluble RNA is not a precursor of ribosomal RNA. The difference in base composition also casts doubt on functional soluble RNA as being a degradation product of ribosomal nucleic acid. It thus seems likely that chloramphenicol stimulates soluble RNA synthesis in resting *S. flexneri* serotype 3.

The demonstrated activity of soluble chloramphenicol-RNA suggests that the stimulatory action is the result, rather than the cause, of the blockage of protein synthesis by the drug. A suppression of protein production may result in the channelling of available nitrogen into RNA (Fraenkel & Neidhardt, 1961). The results thus indicate it is doubtful that chloramphenicol inhibits protein synthesis by inducing the production of physiologically inactive 'abnormal' RNA or by altering the activity of RNA.

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