The Nature of the Proteins in 'Chloramphenicol Particles' from *Escherichia coli* A19 (Hfr *rel met rns*)

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(Received 26 March 1976)

SUMMARY

The unusual particles which accumulate in cell-free extracts from *Escherichia coli* A19 during chloramphenicol inhibition ('chloramphenicol particles') have been isolated by large-scale rate-zonal density gradient ultracentrifugation. The proteins and RNA species composing these particles have been examined.

The rRNA species present are precursor and mature forms of 16S and 23S rRNA which accumulate during inhibition. The proteins prepared directly from the particles give strong multiple immunoprecipitates with antisera specific to 30S and 50S ribosomal proteins. The soluble proteins of the cell prepared in the same manner do not give this immunological reaction. Two-dimensional electrophoresis patterns of the proteins from the 'chloramphenicol particles' strongly resemble those for 30S and 50S ribosomal proteins, i.e. they are predominantly basic low molecular weight proteins, and are dissimilar to the patterns for the soluble proteins of the cell.

It is concluded that the 'chloramphenicol particles' are a heterogeneous group of ribonucleoproteins comprising the bulk of the rRNA accumulating during inhibition in association with variable amounts of some of their corresponding ribosomal proteins. The particles are therefore not artefacts of preparation, as previously thought, but arrested ribosome precursors.

INTRODUCTION

In the study of biogenesis and its associated controls the exceptions to the normal situation frequently provide the key to understanding the normal process. The biosynthesis of ribosomes in bacteria is an example, since in bacteria protein synthesis and RNA synthesis are normally interdependent but there are a number of well studied circumstances in which this interdependence may be broken and RNA synthesis may continue whilst protein synthesis is greatly depressed. These circumstances have clear implications for ribosome biogenesis. It is well known that mutation at the ribonucleic acid control (RC) locus in *Escherichia coli*, from *rel*⁺ (stringent) to *rel* (relaxed), divorces RNA synthesis from protein synthesis in amino-acid auxotrophs deprived of their required amino acid (see Ryan & Borek, 1971, for review). This relaxation of interdependence may also be achieved in both stringent (*rel*⁺) and relaxed (*rel*) strains by adding the antibiotics chloramphenicol or puromycin to growing cultures (Dagley & Sykes, 1959; Nomura & Watson, 1959; Dagley *et al.*, 1962; Sells, 1964; Nomura & Hosokawa, 1965; Hosokawa & Nomura, 1965), by Mg²⁺-starvation of *rel*⁺ amino-acid auxotrophs before withdrawing the amino acid (Nakada & Marquisee, 1965), and by K⁺-deprivation of certain mutants requiring high levels of K⁺ for growth (Ennis & Lubin, 1965*a, b*).

During chloramphenicol inhibition of growing cultures the net accumulation of RNA

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usually halts when the amount of RNA in the cell reaches two to three times the pre-inhibition level. All the normal cellular RNA species continue to be made and in the case of tRNA and rRNA it is mainly their precursor forms which accumulate (Adesnik & Levinthal, 1969; Dean & Sykes, 1974). These rRNA precursors have slightly higher molecular weights than their mature forms found in ribosomes, have different electrophoretic mobilities, and may also be sub-methylated (Lowry & Dahlberg, 1971).

Centrifugal fractionations of crude cell-free extracts from *E. coli* subjected to chloramphenicol inhibition (and all the other circumstances which permit RNA synthesis and depress protein synthesis) show a common but abnormal pattern of slowly sedimenting ribonucleoprotein particles which are very labile and which steadily accumulate as the inhibition proceeds (Dagley *et al.*, 1962; Dagley, Turnock & Wild, 1963; Dean & Sykes, 1974). These particles are usually referred to as 'chloramphenicol (CAL) particles' [or 'relaxed particles', for those from the *rel met* (requirement for L-methionine) strain starved for methionine, or 'puromycin particles' etc.]. These descriptions comprise a heterogeneous group of particles, sedimenting in crude cell-free extracts between 10 and 30S, and containing the bulk of the accumulated precursor rRNA associated with protein (Adesnik & Levinthal, 1969; Dean & Sykes, 1974). The RNA:protein ratios of these particles vary and are, as a population, generally reported to be higher than the ratio for complete mature ribosomes (Nomura & Watson, 1959; Dagley & Sykes, 1959; Hosokawa & Nomura, 1965; Yoshida & Osawa, 1968). 'Chloramphenicol particles' rapidly disappear when the antibiotic is removed from the culture and the accumulated precursor rRNA within the particles is converted into normal 30S and 50S ribosomes without prior degradation, unless 5-fluorouracil has also been incorporated into the RNA during inhibition in which case the RNA is then degraded (Hosokawa & Nomura, 1965; Nakada, 1965; Adesnik & Levinthal, 1969). The evidence therefore favours the view that the rRNA species which accumulate during inhibition are arrested, normal precursor forms.

The origin and fate of the proteins associated with the rRNA in the 'chloramphenicol particles' is much less clear. The majority of the protein components of the particles would appear to be synthesized before the drug inhibition commences (Kurland & Maaløe, 1962; Hosokawa & Nomura, 1965; Yoshida & Osawa, 1968) and could come from a reservoir(s) of pre-existing ribosomal protein or simply be non-specific, general cellular proteins which become associated with the accumulating RNA. The nature of the rRNA and its behaviour on removal of the drug (see above) together with the observations that on recovery rRNA synthesis is temporarily suppressed and certain ribosomal proteins are preferentially synthesized (Davis & Sells, 1969), favour the earlier hypothesis (Dagley *et al.*, 1962; Sykes, 1966) that the various 'particles' are arrested precursors of ribosomes. In these circumstances this hypothesis implies two important points. First, the proteins associated with the rRNA in the 'chloramphenicol particles' must be ribosomal proteins and, secondly, an available source of these proteins must exist or arise in the cells during the inhibition. The supply of these proteins must be sufficient in both amount and specificity to satisfy the minimum requirements for the stabilization of the accumulating precursor rRNA in a variety of early ribosome precursors. In respect of the proteins, the techniques for a direct test of this hypothesis were not immediately available. Indirect tests were made by measuring the level of the free pool of ribosomal proteins in exponentially growing cells on the assumption that this pool was the sole source of ribosomal proteins in the inhibited cells. The two main experimental approaches to this problem gave widely differing results. The direct measurements of the ribosome-free supernatants gave a wider range of values, i.e. 1 to 20 % of the total ribosomal protein in the free pool (Santer *et al.*, 1968; Dalgarno

& Gros, 1968; Voynow & Kurland, 1971; Gupta & Singh, 1972; Subramanian, 1974), than indirect measurements using radioactive pulse-chase techniques, which gave 2 to 4 % in the free pool (Schleif, 1967; Gierer & Gierer, 1968; Gupta & Singh, 1972; Marvaldi *et al.*, 1974; Gausing, 1974). It was therefore generally assumed that the level of the free pool of ribosomal proteins was insufficient to supply the 25 % of total ribosomal protein estimated to be associated with the chloramphenicol particles. Finally on the basis of kinetic, radioactive pulse-chase experiments and experiments involving the mixing of bacterial extracts it was concluded that the 'chloramphenicol particles' were artefacts produced by the random association of the general basic soluble proteins with the accumulated rRNA during the preparation of the cell-free extracts (Schleif, 1968; Yoshida & Osawa, 1968).

The experiments reported in this and the following paper were therefore undertaken to provide a direct test of the nature of the proteins associated with the 'chloramphenicol' and 'relaxed particles' by isolating the proteins from the particles and comparing their immunological and two-dimensional electrophoretic behaviour with ribosomal and non-ribosomal proteins from the same organism. In this paper, the 'chloramphenicol particle' proteins are shown to be ribosomal by these criteria.

METHODS

Organism and growth conditions. *Escherichia coli* A19 (Hfr *met rel rns*) was used throughout these experiments (*rns* designates ribonuclease I negative). It was grown in aerated batch culture in 250 ml volumes in 2 l conical flasks in a gyratory shaker at 30 °C. The medium used was the glucose/mineral salts medium plus methionine described by Dean & Sykes (1974), except that the glucose concentration in the overnight (16 h) culture was set at the limiting concentration of 1.0 g l⁻¹. After overnight growth, more glucose (equivalent to 3.0 g l⁻¹) was added aseptically to each flask and the cultures were harvested approximately 2 h later when they were in the exponential phase and the absorbance of the culture at 540 nm had reached approximately 3.0. The doubling time for the organism in these conditions is 1.5 h. The cultures were rapidly harvested at 20 °C by 5 min centrifuging at 10960 g (av.) in the JA10 rotor of a Beckman J21B centrifuge. At this stage some cell pastes were crushed in a bacteria press at -20 °C (Hughes, 1951) to provide the control cell material i.e. actively growing, fully supplemented exponential-phase cells. For the production of 'chloramphenicol particles', other batches of freshly harvested cells were immediately resuspended in fresh warm (30 °C) glucose (4 g l⁻¹)/mineral salts medium containing methionine (50 µg ml⁻¹) and chloramphenicol (50 µg ml⁻¹; Parke Davies & Co., Hounslow, Middlesex) and incubated for a further 2 h in 250 ml volumes in 2 l flasks in a gyratory shaker at 30 °C. During this time the absorbance of the culture at 540 nm only increased by 28.1 % (average of five experiments). During the incubation, RNA synthesis continues, protein synthesis is suppressed and 'chloramphenicol particles' progressively accumulate within the cells (Dagley & Sykes, 1959; Dean & Sykes, 1974). The cells were again harvested by centrifuging as described above and the cell paste was immediately crushed in a Hughes press (Hughes, 1951). If not used immediately, all the crushed bacterial pastes were stored at -20 °C.

Preparation of cell-free extracts for the bulk isolation of ribosomes and 'chloramphenicol particles'. Crushed bacterial paste (control or chloramphenicol treated; 10 to 12 g) was dispersed by gentle homogenization in 40 to 50 ml buffer containing 10 mM-Tris/HCl pH 7.8, 0.3 mM-MgCl₂, 30 mM-NH₄Cl and 6 mM-β-mercaptoethanol (TMN buffer). The homogenate was centrifuged for 45 min at 54 500 g (av.) at 5 °C in the Spinco 30 angle rotor.

The supernatant, an optically clear, crude, cell-free bacterial extract, was decanted from the cell debris. In the case of chloramphenicol-treated preparations, this extract was examined in the analytical ultracentrifuge (Spinco model E) to ensure the presence of 'chloramphenicol particles' (Dean & Sykes, 1974). The bacterial extracts were then centrifuged at 100000 g (av.) for 4 h at 5 °C in the Spinco SW41-Ti rotor. The supernatant fraction was decanted and retained, and the pelleted fraction was resuspended in 20 ml TMN buffer. The protein concentration in the supernatant was determined by the biuret method using crystalline bovine serum albumin as a standard (Gornall, Bardawill & David, 1949), and the approximate ribosome concentration in the resuspended pelleted fraction was obtained by measuring the absorbance at 260 nm and assuming an $E_{1\text{cm}}^{1\%}$ at 260 nm for ribosomes of 95 (Robinson & Sykes, 1973). These estimations were also used to determine the amount of each fraction loaded in the rate-zonal density gradient ultracentrifugations.

Rate-zonal density gradient ultracentrifugation and the collection of ribosomes and 'chloramphenicol particles'. An MSE A1-14 or Spinco Ti-14 zonal rotor was used in a matching centrifuge and loaded at 3000 rev. min⁻¹ with a 10 to 35 % (w/v) sucrose gradient in TMN buffer by means of an MSE Mark I gradient former. The gradient was hyperbolic with rotor radius (Eikenberry *et al.*, 1970) and had a cushion of 35 % (w/v) sucrose in TMN equal to 10 % of the rotor volume. After loading, the sample was displaced into the rotor with an overlay of 150 ml TMN buffer.

Using the final supernatant fractions (see above) from control or chloramphenicol cell-free extracts, 250 to 350 mg total protein was loaded on to the gradient via a syringe attached to the centre line in a volume of 20 to 30 ml in order to achieve a narrow loading zone and thereby improve the resolution. The TMN buffer overlay (150 ml) was then pumped in via the centre line, and the rotor was filled, capped and centrifuged at 5 °C for 24 h at 30000 rev. min⁻¹ (MSE A1-14 rotor) or 16 h at 36000 rev. min⁻¹ (Spinco Ti-14 rotor) i.e. $\Sigma\omega^2t = 8.19 \times 10^{11}$. In this manner the supernatants from chloramphenicol-treated cells were resolved into three 'chloramphenicol particle' fractions P₁, P₂ and P₃ (see Fig. 2*b*) and the supernatants from control cell preparations gave a single peak (Fig. 1*b*) which was used as a source of the soluble (i.e. non-particle) proteins. The amount of the pelleted fraction (see above) loaded on to the gradients varied between 250 and 350 mg ribosome equivalents (based on absorbance at 260 nm) in 20 ml TMN buffer. The overlay was then injected, and the rotor was filled and capped as before, and then centrifuged for 16 h at 26000 rev. min⁻¹, $\Sigma\omega^2t = 4.27 \times 10^{11}$ (MSE A1-14 or Spinco Ti-14 rotor). In the case of the extracts from chloramphenicol-treated cells these runs provided an additional source of P₁, P₂ and P₃ to be pooled with the corresponding supernatant fractions and the 50S (CAL) ribosomes (see Fig. 2*c*). These samples were also used as a source of the RNA preparations (see below). The pelleted fractions from the exponential-phase cells in the rate-zonal centrifugations were the source of control 50S and 30S ribosomes. These gave the corresponding proteins for the immunological and two-dimensional electrophoresis experiments and rRNA (Fig. 1*c*).

At the conclusion of the centrifugations, the zonal rotor was braked to unloading speed (about 3000 rev. min⁻¹) and the contents of the rotor were progressively displaced from the centre of the rotor by pumping 40 % (w/v) sucrose in TMN to the edge of the rotor. The displaced contents were collected in 14 × 10 ml fractions followed by 106 × 5 ml fractions and stored in ice. The absorbance of each fraction was read at 260 nm, and the gradient profiles (Figs 1, 2) were constructed to determine the fractions to be pooled to provide the samples for further analysis.

Preparation of 50 S and 30 S ribosomal proteins, 'chloramphenicol particle' proteins and soluble proteins from the pooled fractions from zonal centrifugation.

1. *Proteins from 50 S and 30 S ribosomes and 'chloramphenicol particles' P₁, P₂ and P₃.* The appropriate fractions from the zonal centrifugations (see Figs 1 and 2) were pooled and chilled in ice. Solid ammonium sulphate was added to each stirred preparation until the solution was just saturated and the resultant precipitate was collected by centrifuging at 5 °C in the Spinco 30 rotor. This procedure precipitated all the protein in the pooled gradient samples. The precipitates were resuspended in a minimum volume of TMN buffer and dialysed to remove all traces of ammonium sulphate.

The proteins were prepared from each preparation by extensive dialysis against 66 % (v/v) acetic acid containing 0.2 M-magnesium acetate (Hardy *et al.*, 1969; Kaltschmidt & Wittman, 1972). The precipitate of RNA was then centrifuged from the preparations and re-extracted with a small volume of 66 % acetic acid/0.2 M-magnesium acetate for 2 h. After centrifuging, these washings were added to the main preparation. Proteins prepared in this way in acetic acid were stored in the deep-freeze at -20 °C.

2. *Soluble proteins from control cells.* The zonal centrifugations with the supernatant fractions from control cells gave a single prominent peak (Fig. 1*b*) which comprised much of the soluble protein of the cell plus tRNA etc. The peak fractions were pooled and the proteins were prepared from these in two ways: (i) by ammonium-sulphate precipitation, dialysis and acetic-acid extraction as described above; and (ii) by ammonium-sulphate precipitation followed by dialysis against TMN buffer lacking β -mercaptoethanol. Pancreatic ribonuclease (EC. 2.7.7.16; Boehringer) was then added (to a final concentration of 10 μ g ml⁻¹), the preparation was incubated for 10 min at 30 °C and then dialysed for 48 h against changes of TMN buffer lacking β -mercaptoethanol. The preparations of soluble proteins were stored at -20 °C (Kaltschmidt & Wittman, 1972).

Two-dimensional electrophoresis of proteins. Two-dimensional electrophoresis of proteins was carried out following the procedures for ribosomal proteins described by Kaltschmidt & Wittman (1970*a*). The conventional first-dimension cylindrical gel was in 8 % (w/v) acrylamide at pH 8.6, and this gel was embedded on a flat-bed gel of 18 % (w/v) acrylamide run at pH 4.6 for the second dimension. The apparatus used for the second-dimension runs was a small-scale unit accommodating up to three gel beds simultaneously; its design mainly followed that of Mets & Bogorad (1974). The apparatus was cooled by circulating water at a controlled temperature of 7 °C. Using this apparatus the total protein load on the first-dimension gel may be as low as 120 μ g but to maximize the detection of any minor components the load was usually varied between 120 and 250 μ g for the three gels run simultaneously. For electrophoresis, the protein samples prepared as described above were dialysed for 36 h against changes of a solution containing (per l distilled water): 3.2 g boric acid; 0.05 g ethylenediaminetetraacetic acid, disodium salt; 0.1 ml β -mercaptoethanol; and 480.0 g urea. Samples of the preparation (0.1 to 0.3 ml, containing 120 to 250 μ g total protein) were then polymerized into the sample gel section. The first-dimension gels were run for 5.5 h at a constant 5 mA/gel at 7 °C, rinsed briefly in equilibration buffer (Kaltschmidt & Wittman, 1970*a*) and embedded in the second-dimension gel. The second-dimension gels were run for 19 h at a constant 20 V/gel at 7 °C. The gels were finally stained for 2 h in 0.25 % (w/v) naphthalene black 10B (R. Lamb, North Acton, London) in 5 % (v/v) acetic acid and electrophoretically destained in 5 % (v/v) acetic acid in an EC489 destaining unit (EC Apparatus Corp., University City, Philadelphia, U.S.A.).

Isolation and gel electrophoresis of RNA from zonal gradient samples. The samples from the gradient, as indicated in Results, were pooled and the RNA was extracted by shaking with an equal volume of the phenol/*m*-cresol/8-hydroxyquinoline mixture described by Kirby (1956). The RNA was recovered from the aqueous phase and subjected to gel electrophoresis in 3 % (w/v) acrylamide gels following the procedure described by Dean & Sykes (1974). The gels were run in silica tubes so that they could be scanned directly at 260 nm to locate the position of the RNA, using a Pye Unicam SP500 spectrophotometer fitted with a Gilford linear transport accessory.

Preparation of immune sera to 50S and 30S ribosomal proteins. The proteins prepared from control 50S and 30S ribosomes, as described above, were dialysed against 1 M-Tris/HCl pH 7.4 and the final preparation was adjusted so that ribosomal protein content was approximately 2.0 mg ml⁻¹. A sample (0.5 ml) of the 50S and 30S ribosomal protein preparation was thoroughly mixed with 0.5 ml Freund's complete adjuvant (Difco) and the mixture was injected intramuscularly in the flank of a female rabbit (which had previously been test bled to provide a source of non-immune serum). After 22 days, the rabbit was given an intravenous injection (via an ear vein) of the appropriate protein preparation plus adjuvant and thereafter intramuscular injections at regular intervals over a period of 4 months, during which time the antigen protein was raised to 2.0 mg per injection. Antibodies to the respective ribosomal proteins were detected, by Ouchterlony immunodiffusion tests, in sera from test bleeds 7 weeks after commencing this programme over injection. After 4 months, 25 ml whole blood was taken via the ear vein of each rabbit. The blood was allowed to clot for 1 h at room temperature and then left overnight at 5 °C. The serum was carefully removed and centrifuged at 13583 *g* (av.) for 15 min in the Spinco angle 50 rotor at 5 °C. The immunoglobulin fraction from each batch of antiserum was precipitated by adding 15.6 g ammonium sulphate to each 50 ml serum with stirring at 5 °C. The mixture was stirred for a further 30 min and the precipitate of immunoglobulins was collected by centrifuging for 10 min at 8720 *g* (av.) and 5 °C. The precipitate was redissolved in 0.5 original vol. 1 M-Tris/HCl pH 7.4 and dialysed against changes of this buffer at 5 °C to remove all traces of ammonium sulphate. These preparations of antibodies to 50S and 30S ribosomal proteins and the non-immune sera were stored at -20 °C in small volumes, and thawed for use as required.

Immunodiffusion analysis. Clean microscope slides (7.6 × 2.5 cm) were skimmed with 1 % (w/v) Davis agar in 1 M-Tris/HCl pH 7.4, and then coated with a 2 to 3 mm layer of this agar solution using the Gelman Immunoframe (Gelman Instrument Co., Ann Arbor, Michigan, U.S.A.). When set, a pattern of holes consisting of a central well surrounded by a circle of six equal wells was punched in the agar surface with a Gelman Immunodiffusion punch set (no. 51449). The central well was filled with a drop(s) of a solution of the prepared proteins (50S, 30S or soluble proteins) containing 1.5 to 2.0 mg protein ml⁻¹ in 1 M-Tris/HCl pH 7.4. Undiluted and serial dilutions of the ammonium-sulphate precipitated antisera preparations were placed in the surrounding holes in a clockwise manner. After 72 h in an enclosed moist atmosphere at 37 °C, the residual proteins were washed from the wells by two changes of 0.9 % (w/v) NaCl followed by distilled water rinses. The slides were dried by overlaying with fibre-free filter paper (Evans, Adlard & Co., Winchcombe, Gloucestershire) at 37 °C for 2 h, and then stained in 0.2 % (w/v) Coomassie blue B.R. (R. Lamb, North Acton, London) dissolved in methanol/water/acetic acid (5:5:7, by vol.) for 15 min. They were destained by rinsing in methanol/water/acetic acid and then water before drying to reveal the immunoprecipitate.

Fig. 1. Analytical schlieren and zonal ultracentrifuge gradient absorbance profiles for extracts from exponential-phase cells of *E. coli* A19. Sedimentation is from left to right.

(a) Analytical ultracentrifuge schlieren diagram for the crude cell-free extract. Protein concentration 10.0 mg ml^{-1} ; temperature 19.1°C ; bar angle 60° ; photograph taken after 20 min at $50740 \text{ rev. min}^{-1}$. From right to left, the sedimenting boundaries correspond to 50S and 30S ribosomes and the cell supernatant i.e. source of non-ribosomal soluble proteins.

(b) Rate-zonal ultracentrifuge density gradient absorbance profile at 260 nm (continuous line) for the supernatant fraction derived from (a). The samples from the gradient pooled to provide the source of soluble proteins are indicated. Run conditions: Spinco Ti-14 rotor; load 260 mg total protein; 16 h at $36000 \text{ rev. min}^{-1}$; 5°C ; gradient 10 to 35 % (w/v) sucrose in TMN buffer hyperbolic with radius (dashed line indicates gradient at end of run).

(c) Rate-zonal ultracentrifuge density gradient absorbance profile at 260 nm (continuous line) for the pellet fraction derived from (a). The samples from the gradient pooled to provide 30S and 50S ribosomes (and their rRNA and proteins) are indicated. Run conditions: MSE A1-14 rotor; load 360 mg ribosome equivalents; 16 h at $26000 \text{ rev. min}^{-1}$; 5°C ; gradient 10 to 35 % (w/v) sucrose in TMN buffer hyperbolic with radius (dashed line indicates gradient at end of run).

Abbreviations. m16, m23, p16 and p23 refer to mature and precursor species of 16S and 23S rRNA. 30S proteins and 50S proteins refer to proteins derived from 30S and 50S ribosomal sub-units.

RESULTS

Zonal and analytical ultracentrifuge separations

Figure 1(a) shows the analytical ultracentrifuge diagram for a crude cell-free extract from fully supplemented exponential-phase cells of *E. coli* A19. Figure 1(b, c) shows the profiles for the supernatant and pellet fractions respectively obtained from this crude extract after

Fig. 2. Analytical schlieren and zonal ultracentrifuge gradient absorbance profiles for extracts from chloramphenicol-inhibited *E. coli* A19 cells. Sedimentation is from left to right.

(a) Analytical ultracentrifuge schlieren diagram for crude cell-free extracts after 2 h inhibition. Protein concentration 10.0 mg ml^{-1} ; temperature 20.5°C ; bar angle 30° ; photograph taken after 20 min at $44\,770 \text{ rev. min}^{-1}$. The 'chloramphenicol particles' are seen, by comparison with Fig. 1(a), to be sedimenting between 50S ribosomes and the soluble protein boundary.

(b) Rate-zonal ultracentrifuge density gradient absorbance profile at 260 nm (continuous line) for the supernatant fraction derived from (a). The samples from the gradient pooled to provide 'chloramphenicol particles' P1, P2 and P3 are indicated. Run conditions: MSE A1-14 rotor; load 332 mg total protein; 24 h at $30\,000 \text{ rev. min}^{-1}$; 5°C ; gradient 10 to 35% (w/v) sucrose in TMN buffer hyperbolic with radius (dashed line indicates gradient at end of run).

(c) Rate-zonal ultracentrifuge density gradient absorbance profile at 260 nm (continuous line) for the pellet fraction from (a). The samples from the gradient pooled to provide additional P1, P2 and P3 material are shown together with those for 50S (CAL) ribosomes. Run conditions: Spinco Ti-14 rotor; load 300 mg ribosome equivalents; 16 h at $26\,000 \text{ rev. min}^{-1}$; 5°C ; gradient 10 to 35% (w/v) sucrose in TMN buffer hyperbolic with radius (dashed line indicates gradient at end of run).

their separation in a sucrose density gradient in the zonal ultracentrifuge (see Methods). The single soluble protein boundary and the gradient samples pooled to provide this material are shown in Fig. 1(b). The samples pooled to provide control 30S and 50S ribosome material (proteins and rRNA) are shown in Fig. 1(c).

Figure 2(a) shows the analytical ultracentrifuge pattern for a crude cell-free extract from *E. coli* A19 cells incubated for 2 h in the presence of chloramphenicol ($50 \mu\text{g ml}^{-1}$). The 'chloramphenicol particles' which accumulated during the period of inhibition are clearly seen (compare Figs 1a and 2a). Figure 2(b) shows the profile of the sucrose density gradient zonal ultracentrifuge separation of the supernatant fraction from the cell-free extract and the samples pooled to provide 'chloramphenicol particles' P1, P2 and P3. Figure 2(c) shows the profile for the corresponding centrifugation of the pellet fraction from which further

Fig. 3. Absorbance profiles at 260 nm for the electrophoretic separation of rRNA preparations in polyacrylamide gels. The rRNA preparations were extracted from the cell-free extracts and gradient fractions, electrophoresed and scanned as described in Methods. (a) Total rRNA (30 μ g) from crude cell-free extracts of exponential-phase cells of *E. coli* A19. (b) Total rRNA (50 μ g) from crude cell-free extracts of exponential-phase cells of *E. coli* A19 2 h after transfer to fresh medium containing chloramphenicol (100 μ g ml⁻¹). (c) rRNA (28 μ g) from 50S (CAL) ribosomes recovered from the zonal ultracentrifuge (see Fig. 2c). (d) rRNA (40 μ g) from 'chloramphenicol particle' region P1 (see Fig. 2b, c). (e) rRNA (34 μ g) from 'chloramphenicol particle' region P2 (Fig. 2b, c). (f) rRNA (30 μ g) from 'chloramphenicol particle' region P3 (Fig. 2b, c).

samples of P1, P2 and P3 were obtained and pooled with those from the supernatant. The samples for 50S (CAL) ribosome preparations were also taken from this zonal centrifugation (Fig. 2c).

rRNA species present in the samples from control cells and cells treated with chloramphenicol

The gel electrophoretograms of the total rRNA prepared directly from crude cell-free extracts from control exponential-phase cells and chloramphenicol-treated cells are shown in Fig. 3. The profile for the control preparation (Fig. 3a) reveals a preponderance of m23S and m16S rRNA and little p16S rRNA. This pattern is consistent with the analytical and zonal ultracentrifugation profiles (Fig. 1a, c) which show only mature 50S and 30S ribosomes. The electrophoretogram for the total rRNA prepared from the cells treated with chloramphenicol (Fig. 3b) shows larger amounts of p16S rRNA, in agreement with previous observations (Dean & Sykes, 1974). This accumulation of p16S rRNA parallels the accumulation of 'chloramphenicol particles' seen in the ultracentrifuge (Fig. 2). p23S rRNA is very poorly resolved from the bulk of m23S rRNA in these gels.

The gel electrophoretograms for the rRNA prepared from the 'chloramphenicol particles' P1, P2 and P3 and from the 50S (CAL) ribosomes recovered from the zonal density gradients

show that the accumulating precursor rRNA is mainly associated with the 'chloramphenicol particles' (Fig. 3*c, d, e, f*). Figure 3(*c*) shows that the usual single m23S rRNA species is still present and intact in 50S ribosomes taken from chloramphenicol-treated cells. The 'chloramphenicol particle' region samples P₁, P₂ and P₃ contain different proportions of p23S, m23S, p16S and m16S rRNA (Fig. 3*d, e, f*). Particle P₁ (Fig. 3*d*) is a mixture of species with a preponderance of p23S and p16S rRNA, compared with P₂ (Fig. 3*e*) and P₃ (Fig. 3*f*). The rRNA preparations from P₁ and to a lesser extent P₂ invariably showed evidence of breakdown of the RNA which was not observed in the simultaneous preparation from P₃ or the corresponding preparation from methionine-starved cells (Sykes, Metcalf & Pickering, 1977). The proportion of m16S rRNA is greater in P₂ than P₃ and in the latter, which encompasses the 30S ribosome region, there is no evidence of p16S rRNA and m16S rRNA is the dominant species. m23S rRNA is also present in P₂ and P₃ and p23S rRNA in P₁. These are the rRNA species of 50S precursors and their presence in these 'chloramphenicol particles' is consistent with the evidence from immunological and protein electrophoresis experiments detecting 50S proteins in the particles.

From these electrophoretograms we conclude that the bulk of the p16S rRNA in chloramphenicol-treated cells is associated with the 'chloramphenicol particles' P₁, P₂ and P₃. Furthermore, as prepared for these experiments, each particle region is complex comprising the various precursor and r-mature RNA species in different proportions. The rRNA species in the more slowly sedimenting parts of the region (P₁ and P₂) appear to be relatively unstable. It may be noted that fractions containing only one of the rRNA species noted above may be recovered from the gradients of the particle region if finer sampling is used than the pooling of many samples represented by P₁, P₂ or P₃. Unfortunately it was necessary to have the bulked samples to provide sufficient material for the subsequent immunological and electrophoretic identification of the proteins.

Immunological properties of the proteins from 'chloramphenicol particles'

The non-immune sera taken from rabbits before immunization gave a very slight immune reaction with 50S proteins when undiluted and a slightly stronger reaction against 30S proteins. These slight responses to ribosomal proteins are probably the result of previous exposure of the rabbits to bacterial infection. In contrast the sera taken at the end of the immunization period gave strong multiple immunoprecipitates at various dilutions with their corresponding antigens, i.e. 30S or 50S proteins. The immunization programme had clearly elicited the production of antibodies to the ribosomal proteins. The 30S and 50S proteins of *E. coli* A19 are known to be immunologically distinct. This was essentially confirmed since the 50S proteins did not react with antisera developed against 30S proteins. However, the preparations of 30S proteins gave a strong reaction with antisera to 50S proteins. In view of the established individuality of the ribosomal proteins this latter reaction must have been caused by the presence of some 50S proteins in the 30S preparations. The most likely reason for this is the presence of a known 50S precursor on the leading edge of the 30S ribosome boundary in the gradients (Nierhaus, Bordasch & Homann, 1973; Lindahl, 1975) coupled with incomplete resolution in the zonal ultracentrifuge.

In sharp contrast to the above observations, the antisera to 50S and 30S proteins gave only minimal immune reactions with the proteins prepared by ribonuclease or acetic-acid treatment (see Methods) from the soluble fraction of control cultures. The immunization programme had therefore raised antisera specific to 50S and 30S ribosomal proteins which did not react with general cellular proteins extracted by the same methods as used for the ribosomal proteins.

Fig. 4. Immunoprecipitates formed during double diffusion in one dimension between proteins prepared from 'chloramphenicol particles' P1, P2 and P3 and antisera specific to 50S or 30S ribosomal proteins. Undiluted antiserum to 50S (left) or 30S proteins (right) was in the topmost well (12 o'clock) and serial dilutions (1:1) were arranged clockwise in the surrounding wells. Proteins were in the centre well: (a) proteins from P1; (b) proteins from P2; and (c) proteins from P3.

When the specific antisera were tested against the proteins obtained from 'chloramphenicol particles' P1, P2 and P3, all gave strong multiple immunoprecipitates at all dilutions of the respective antisera (Fig. 4). This result unambiguously establishes that some of the proteins associated with the 'chloramphenicol particles' must be ribosomal. Furthermore both 50S and 30S proteins are present in the particle region. In addition the very weak reaction given by the soluble protein fraction from exponential-phase cells to both 30S and 50S antisera confirms the low level of the free ribosomal protein pool in growing cells (Gausung, 1974).

Two-dimensional electrophoresis of proteins from 50S ribosomes, 30S ribosomes and soluble proteins from control cells, and 50S (CAL) ribosomes and particles P1, P2 and P3 from chloramphenicol-inhibited cells

The two-dimensional gel electrophoretic separation of the soluble protein fraction prepared via acetic-acid or ribonuclease extraction from zonal gradient samples of exponentially growing *E. coli* A19 (see Methods and Fig. 1a, b) is shown in Fig. 5(a). With

Fig. 5. Two-dimensional electrophoresis patterns for the proteins prepared from the soluble fraction, 30S and 50S ribosomes of exponential-phase cells of *E. coli* A19 and the 'chloramphenicol particles'. All proteins were prepared by the acetic-acid extraction procedure (see Methods), and electrophoresis followed the procedure of Kaltschmidt & Wittman (1970a). (a) Soluble proteins prepared from exponential-phase cells, 216 μg total protein loaded. (b) 30S ribosomal proteins from exponential-phase cells, 250 μg total protein loaded. (c) 50S ribosomal proteins from exponential-phase cells, 180 μg total protein loaded. (d) Proteins from 'chloramphenicol particle' P1, 190 μg total protein loaded. (e) Proteins from 'chloramphenicol particle' P2, 180 μg total protein loaded. (f) Proteins from 'chloramphenicol particle' P3, 160 μg total protein loaded.

one exception, all the proteins in this preparation have isoelectric points below pH 8.6 and are only weakly acidic since they barely migrate from the first-dimension sample gel. They are also of relatively large molecular weight compared with ribosomal proteins since they do not migrate far into the second-dimension gel, for example, the position of the slowest migrating spot in the corresponding electrophoresis of the control 30S proteins (Fig. 5*b*), which is also weakly acidic with a $pI < 8.6$, i.e. S1, has a molecular weight of 65 000 which is unusually high for ribosomal proteins. Therefore using identical extraction and separation procedures to those used to isolate the proteins from the ribosomes and particles, the proteins prepared from the soluble fraction of exponentially growing *E. coli* A19 cell extracts were acidic and of relatively large molecular weight. This conclusion is in agreement with the observations of Subramanian (1974) who used different conditions of electrophoresis in two-dimensional gel systems. It is important to note that although the ribonuclease method of preparation of the soluble proteins gave a better yield, the number and distribution of the proteins in the second-dimension gel was the same as that found with the method using acetic acid. In both preparations some proteins invariably remained in the sample gel. Thus the acetic-acid procedure did not selectively extract a special class of proteins from the soluble fraction and it is therefore reasonable to compare the two-dimensional electrophoresis patterns of the proteins extracted by this method (see Kaltschmidt & Wittmann, 1972).

The electrophoretic pattern for the soluble proteins is in sharp contrast to the corresponding patterns for the ribosomal proteins prepared in the same way from 50S and 30S ribosomes from exponentially growing *E. coli* A19 (Fig. 5*b, c*). Migration in the first dimension indicates that most proteins from 50S ribosomes are basic with pI values above 8.6, although at least three distinct spots are acidic (Fig. 5*c*), and most of the proteins are of relatively low molecular weight as indicated by their strong migration in the second-dimension gel. This pattern with at least 26 resolved spots shows many of the characteristics of 50S proteins from *E. coli* A19 noted by others (see, for example, Wittmann, 1974) and, most significantly, it is readily distinguished in every aspect from the pattern of general cellular proteins (compare Fig. 5*c* and *a*). The same conclusion is drawn from the pattern of ribosomal proteins from normal 30S ribosomes (Fig. 5*b*). Although only 14 of these are resolved, they are again predominantly basic, low molecular weight proteins. At least three of them are acidic but all are distinguishable from similar soluble proteins by their position in the second-dimension gel.

The profile of absorbance at 280 nm for the fractions taken from the zonal ultracentrifuge parallels that at 260 nm (Figs 1, 2). This and the positive biuret test on the fractions P1, P2 and P3 establish the presence of protein throughout the 'chloramphenicol particle' region. This protein was isolated in good yield from each of the original pooled fractions P1, P2 and P3 by the acetic-acid extraction described in Methods. The two-dimensional gel separations of the proteins from P1, P2 and P3 are shown in Fig. 5(*d, e, f*). Comparison of these with the patterns for the soluble and control ribosomal proteins (Fig. 5*a, b, c*) indicates that the proteins in the particles are quite distinct from the soluble proteins (Fig. 5*a*) and strongly resemble the ribosomal proteins. The majority of the proteins associated with the particles have pI values above 8.6 and are of relatively low molecular weight. Furthermore, the number of protein components associated with the rRNA in the particles increases with the increasing sedimentation coefficient of the fraction (P1 $\rightarrow$ P3). The proteins from P1 are resolved into at least 11 basic components (five strongly represented) and three acidic components, none of which corresponds to the spots in the pattern for the soluble proteins. This is also true for the larger number of proteins associated with P2: a minimum of

14 basic spots (seven strongly represented) and four acidic spots are resolved. P₃ shows 16 basic spots, all strongly represented, and four acidic spots. These two-dimensional electrophoretograms therefore confirm the conclusion from the immunological experiments that the proteins associated with the rRNA species in the 'chloramphenicol particles' are ribosomal. Furthermore, the electrophoretograms also show that the majority of the proteins associated with the particles are ribosomal in character and few, if any, resemble the soluble proteins of the cell.

Unfortunately a precise identification of the ribosomal proteins associated with the particle regions according to the nomenclature of Kaltschmidt & Wittman (1970*b*) is not possible. This will require a much finer sampling of the zonal density gradients to yield samples containing one rRNA species and its associated proteins. This has been achieved for the rRNA but the amount of associated protein is too small for our present electrophoretic procedures. Despite this it is possible to superimpose the electrophoretograms for the particles on those from normal 50S and 30S proteins and observe that many of the well-defined spots in each are co-migrating. This is particularly true for P₃ in which there is a strong correlation with the well-defined spots in both the 50S and 30S protein patterns. This observation is consistent with the presence of m16S and m23S rRNA and the greater number of ribosomal proteins found in this most rapidly sedimenting fraction.

Since the 23S rRNA in the 50S (CAL) ribosomes apparently remains intact throughout the inhibition (see Fig. 3*c*) it is possible that a source of some of the 50S proteins associated with the particles may be the selective dissociation of proteins from 50S during the inhibition. However, a comparison of the electrophoretograms of the proteins from 50S (CAL) and control 50S ribosomes did not reveal any significant loss of protein components from the residual 50S (CAL) ribosomes. The total breakdown of 50S (or 30S) ribosomes during chloramphenicol inhibition reported in other experiments (Dagley & Sykes, 1959; Nomura & Watson, 1959; Lefkovits & di Girolamo, 1969; Young & Nakada, 1971) would not be detected in these experiments, particularly if the ribosomal proteins arising from this were conserved and immediately associated in the particles with the nascent precursor rRNA.

DISCUSSION

Chloramphenicol treatment of growing *E. coli* A19 results in an immediate suppression of protein synthesis and a continuation/stimulation of RNA synthesis. As the inhibition proceeds there is a steady accumulation of 'chloramphenicol particles' in the cell-free extracts sedimenting at 10 to 30S. The experiments reported in this paper show that the rRNA which accumulates during the inhibition is to be found within these particles in the form of mature and precursor forms of 16S and 23S rRNA, precursor 16S rRNA being particularly evident. Protein is associated with these particles, but it has been claimed that they are artefacts formed by the random association of the accumulating rRNA and the soluble proteins of the cell (Schleif, 1968; Yoshida & Osawa, 1968). The immunological and electrophoretic properties of the proteins reported in this paper unequivocally establish that the particle proteins are entirely ribosomal in character and in no way do they equate with the soluble proteins. The 'chloramphenicol particles', even at high chloramphenicol concentrations, are therefore a heterogeneous group of ribonucleoproteins consisting of the accumulating normal precursor and mature rRNA species (16S and 23S) in association with varying amounts of some of their corresponding ribosomal proteins. These properties, together with their increasing numbers of ribosomal proteins as their sedimentation coefficient increases, indicate that the particles are arrested ribosome precursors and not

artefacts. This conclusion is reinforced by the observation that the precursor rRNA which accumulates is not degraded on the release of chloramphenicol inhibition but is directly converted to mature rRNA within normal ribosomes (Nomura & Hosokawa, 1965). Furthermore, in the early recovery period from inhibition, RNA synthesis is depressed and protein synthesis resumes and is dominated by the preferential synthesis of a limited number of ribosomal proteins (Davis & Sells, 1969).

The immunological and protein electrophoresis experiments with the soluble proteins of the cell indicate very low levels of free ribosomal proteins in exponential-phase cells. This is in agreement with other experiments to determine the level of the free pool of ribosomal proteins at various growth rates (Gausung, 1974). There was no clear evidence from the ribosomal protein pattern of 50S (CAL) ribosomes to suggest that ribosomes remaining intact throughout the inhibition provide a select group of proteins for the particles, for example, the 'split proteins' detached readily from ribosomes by adjustment in the cation balance. The implications of these observations are considered in the following paper in conjunction with similar results obtained with the 'relaxed particles' formed during methionine-starvation of this strain of *E. coli* A19.

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