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Inhibition of Bacterial Growth by Metal Salts

THE ACCUMULATION OF RIBONUCLEIC ACID DURING INHIBITION OF *ESCHERICHIA COLI* BY COBALT CHLORIDE

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During inhibition of the growth of *Escherichia coli* by cobalt chloride protein synthesis was decreased more than the synthesis of RNA. Three species of particle accumulated during the incubation. These had sedimentation coefficients of about 44s, 33s and 23s in tris buffer containing 10 mM-magnesium acetate and 100 mM-potassium chloride, but their sedimentation properties were susceptible to changes in buffer composition. The particles contained RNA but were more readily degraded by ribonuclease than were the ribosomes. RNA isolated from the particles differed slightly in sedimentation properties from the major species of ribosomal RNA. The particles are likely to be closely related to ribosome precursors that have been detected in other circumstances. Changes in the polyribosome fraction during inhibition by cobalt chloride, nickel chloride and chloramphenicol provided further evidence that inhibition by Co^{2+} involves specific effects on the protein-synthesizing machinery.

The preceding paper (Blundell & Wild, 1969) examined the synthesis of RNA and protein during inhibition of bacterial growth by toxic metal salts and showed that the action of cobalt chloride was different from that of the other salts that were tested. Protein synthesis was more affected by Co^{2+} than was RNA synthesis, so that RNA accumulated during inhibition. This paper investigates the nature of the RNA that is made in these circumstances. A major portion is shown to be in three species of particle whose RNA differs slightly in its sedimentation properties from ribosomal RNA; the particles are also distinct from ribosomes in their sensitivity to ribonuclease and in their sedimentation behaviour in different buffers. The status of the particles is considered and their accumulation related to other circumstances in which bacteria make an excess of RNA.

EXPERIMENTAL

Growth of bacteria. Escherichia coli strain M.R.E. 600 was grown, with a mean generation time of about 50 min. at 37°, in a tris–salts–glucose medium as described in the preceding paper (Blundell & Wild, 1969). Cultures were incubated overnight in media containing a limiting concentration (7 mg./l.) of glucose to E_{450} about 0·015; the following morning, 2 g. of glucose/l. was added and experiments were started when the extinction was 0·20–0·25.

In several experiments, cells were used that had incorporated [^{3}H]uracil and [^{14}C]uracil into RNA. Glucose was added to an overnight culture as described above; 15 min. later, 100–130 nc of [^{3}H]uracil/ml. was added, and after a further 45 min. 15 μg. of non-radioactive uracil/ml. The cells were then allowed to grow to the chosen extinction. This procedure allowed the detection of preformed stable RNA in lysates of harvested cells. To label the RNA made in the presence of cobalt chloride or other inhibitors, 100 nc of [^{14}C]uracil/ml. was then added to the culture with the inhibitor.

Lysis of cells. The method used was essentially that of Godson (1967). Cultures (25–50 ml.) were chilled rapidly by the addition of 0·5 vol. of crushed ice. Thereafter all operations were carried out at 0°. The cells were collected by centrifuging and resuspended in 0·4 ml. of 25% (w/v) sucrose in 10 mM-tris chloride buffer, pH 8·1. A solution (0·1 ml.) of 0·42 mg. of lysozyme/ml. and 1·35 mg. of disodium EDTA/ml. in 0·12 M-tris chloride buffer, pH 8·1, was added and the mixture was allowed to stand for 45 sec. The suspension was then added to 0·5 ml. of a mixture containing 1% (w/v) Brij 58, 0·04% sodium deoxycholate, 8 mM- or 20 mM-$MgSO_4$ and 20 μg. of deoxyribonuclease/ml. in 40 mM-tris chloride buffer, pH 8·1; 20 mM-$MgSO_4$ was used in the mixture when subsequent sucrose-density-gradient centrifugation was in buffers containing 10 mM-magnesium acetate (see below). Lysis (assessed visually) was complete within about 5 min. More than 90% of the radioactive RNA of lysed cells was recovered in fractions after centrifuging in sucrose density gradients.

Sucrose-density-gradient analysis. The following buffers were used, all at pH 7·4: (i) 10 mM-tris chloride buffer–10 mM-magnesium acetate–100 mM-KCl ('THMK'); (ii) 10 mM-tris chloride buffer–10 mM-magnesium acetate ('THM'); (iii) 10 mM-tris chloride buffer–2 mM-magnesium acetate–100 mM-KCl ('TLMK'); (iv) 10 mM-tris chloride buffer–0·1 mM-magnesium acetate ('TM').

Linear sucrose gradients (15–30%, w/w) in 5·0 ml. of the appropriate buffer were prepared as described by Britten & Roberts (1960). Exponential concave sucrose gradients (15–50%, w/w) (Bock & Ling, 1954) were prepared as follows: 4·0 ml. of 50% (w/w) sucrose in buffer was stirred in a small glass bottle through whose screw-cap was fitted the tip of a

burette and an outlet of thin polythene tubing. Buffer (5ml.) from the burette was allowed to enter the bottle and the same volume of effluent collected.

Gradients were usually layered with 0·1ml. of a preparation of lysed cells and centrifuged at 4° and 124000*g* in the SW39 rotor of a Spinco model L ultracentrifuge. After centrifuging about 40 fractions were collected from the bottom of a tube into 2ml. of ice-cold 5% (w/v) trichloroacetic acid for the assay of radioactivity. Fractions were filtered on Whatman GF/B glass-fibre circles and washed with 5% (w/v) trichloroacetic acid at 0°. The circles were then transferred to scintillation vials and dried at 80° for 1hr. in a vacuum oven. For some analyses another procedure, which gave the same results, was used. Fractions were then collected directly on to strips (approx. 23cm.× 1·5cm.) of Whatman GF82 glass-fibre paper; the strips were dried at 37° for 30min. and immersed in 5% (w/v) trichloroacetic acid at 0° for 10min. They were rinsed thoroughly with 5% trichloroacetic acid at 0° and then with ethanol before being dried for 15min. at 80° in a vacuum oven. The strips were cut up and pieces containing the fractions placed in scintillation vials.

Analysis of RNA. Lysates of cells or fractions from gradients were incubated with 0·1 vol. of 10% (w/v) sodium dodecyl sulphate for 5min. at 37° (Kurland, 1960). Portions of the preparations were then centrifuged on linear 15–30% (w/w) sucrose gradients made in 10mM-sodium acetate buffer, pH4·6, containing 100mM-NaCl. Conditions of centrifuging and methods of collection were as described above.

Other methods. The incorporation of radioactivity into whole cells and radioactivities of fractions from density gradients were measured as described in the preceding paper (Blundell & Wild, 1969).

Chemicals. [5-^{3}H]Uracil, [2-^{14}C]uracil, [^{35}S]sulphate and [^{32}P]phosphate were from The Radiochemical Centre, Amersham, Bucks., lysozyme and chloramphenicol were from the Sigma (London) Chemical Co., London S.W.6, ribonuclease was from Koch–Light Laboratories Ltd., Colnbrook, Bucks., deoxyribonuclease was from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., and Brij 58 from Honeywill and Stein Ltd., Carshalton, Surrey.

RESULTS

Oversynthesis of RNA during inhibition by cobalt chloride. The experiments described in this paper were with exponentially growing cultures of *E. coli* with E_{450} 0·20–0·25. The inhibition produced by a given concentration of cobalt chloride was then somewhat less than in the experiments at lower cell densities described previously (Blundell & Wild, 1969). Table 1 shows that there was slight oversynthesis of RNA during incubation with 0·03mM-cobalt chloride; this was partially a consequence of a small but consistent stimulation of the rate of synthesis of RNA during the first 30min. of inhibition. Although the syntheses of RNA and protein were both increasingly inhibited by higher concentrations of cobalt chloride, the relative oversynthesis of RNA was then greater; during inhibition by 0·3mM-cobalt chloride, the increase in RNA per unit increase in protein was twice that in the control culture. Most of the experiments that follow investigate the nature of the RNA made during inhibition for 30min. by 0·3mM-cobalt chloride; in the experiment of Table 1, the RNA and protein contents per millilitre of culture increased by 37% and 17% respectively in this time.

Table 1. *Synthesis of RNA and protein during inhibition by cobalt chloride*

Portions (50ml.) of a culture of *E. coli* (E_{450} 0·20) containing 15μg. of uracil/ml. were incubated with 100nc of [^{3}H]uracil/ml., 70nc of [^{35}S]sulphate/ml. and $CoCl_2$ at the stated concentrations. Samples (1·0ml.) were taken at intervals for measurement of the radioactivity incorporated into RNA and protein. The method used and a detailed explanation of the IC ratio, which is a measure of the oversynthesis of RNA relative to protein, are given in the preceding paper (Blundell & Wild, 1969).

Concn. of $CoCl_2$ (mM)	Time (min.) ...	15	30	45
		Radioactivity (c.p.m./ml.) incorporated into RNA		
0		321	791	1311
0·03		371	813	1218
0·1		318	662	910
0·3		284	487	556
		Radioactivity (c.p.m./ml.) incorporated into protein		
0		155	361	647
0·03		157	310	544
0·1		108	216	343
0·3		71	108	123
		IC ratio		
0·03		1·2	1·3	1·1
0·1		1·4	1·5	1·3
0·3		2·0	2·2	2·2

Sucrose-density-gradient analysis of lysed cells. The nature of components containing the RNA made during the inhibition by Co^{2+} was investigated. Cells were grown in medium containing [^{3}H]uracil, as described in the Experimental section, to label stable forms of RNA; cobalt chloride (final concentration 0·3mM) and [^{14}C]uracil were then added. After 30min. the cells were harvested, lysed and portions of the lysate examined by centrifuging in exponential sucrose density gradients in THMK buffer. After centrifuging for 45min. the stable preformed RNA was largely present in fractions comprising polyribosomes, 70s ribosomes and transfer RNA (Fig. 1*a*). The presence of polysomes after the strong inhibition of RNA and protein synthesis produced by Co^{2+} is noteworthy and is considered in more detail below. Some of the ^{14}C-labelled RNA made during inhibition was also in the polyribosomes and transfer RNA but another portion was in a region of the gradient normally

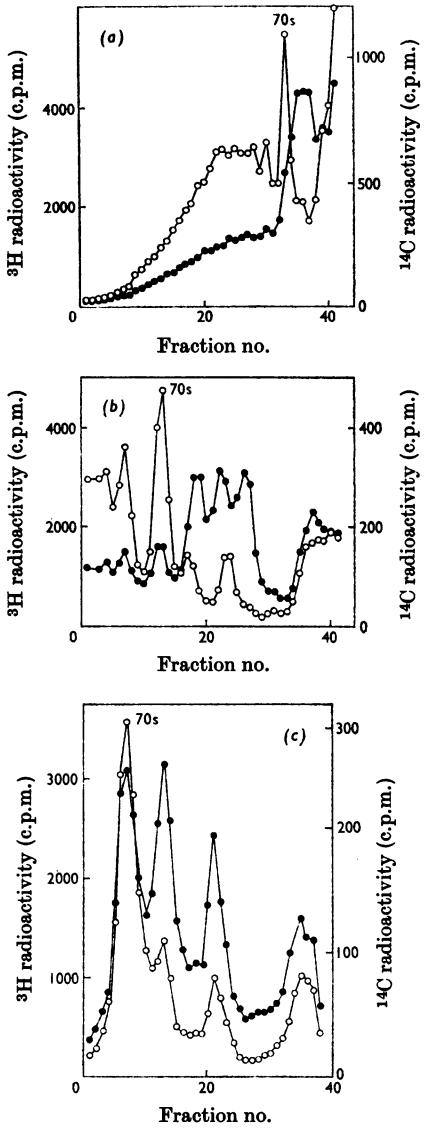

Fig. 1. Sucrose-density-gradient analyses of lysed *E. coli* after inhibition by $CoCl_2$. The general procedures are described in the text. For (*a*) and (*b*), a culture (50ml.; E_{450} 0·24) already labelled with [^{3}H]uracil was inhibited by 0·3mM-$CoCl_2$ for 30min. in the presence of 100nc of [^{14}C]-uracil/ml. The cells were collected and lysed and portions (0·1ml.) were layered on to exponential sucrose density gradients made in THMK buffer. Centrifuging was for (*a*) 45min. and (*b*) 4hr. For (*c*), a culture (50ml.; E_{450} 0·23) already labelled with [^{3}H]uracil was incubated for 10min. with 130nc of [^{14}C]uracil/ml. in the absence of inhibitor. The cells were harvested and lysed, and a portion (0·1ml.) was layered on to a linear sucrose density gradient made in THMK buffer. Centrifuging was for 4hr. Fractions (2 drops) were collected for the measurement of ^{3}H radioactivity (○) and ^{14}C radioactivity (●) incorporated into material insoluble in 5% trichloroacetic acid.

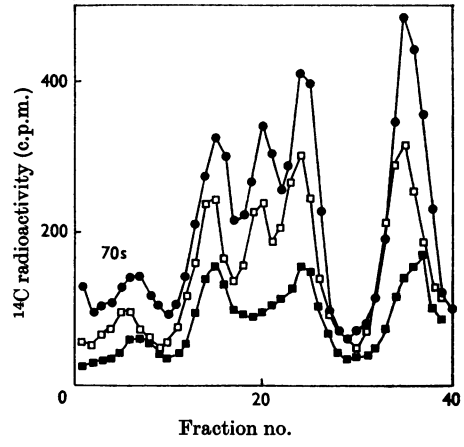

Fig. 2. Time-course of synthesis of 'cobalt particles'. The general procedures are described in the text. A culture (150ml.; E_{450} 0·24) was labelled with 130nc of [^{14}C]-uracil/ml. in the presence of 0·3mM-$CoCl_2$. Samples (50ml.) were taken after 10, 20 and 30min. and the cells harvested and lysed. Portions (0·1ml.) of the lysates were layered separately on to linear sucrose density gradients made in THMK buffer. Centrifuging was for 4hr. Fractions (2 drops) were collected for the measurement of radioactivity incorporated into material insoluble in 5% trichloroacetic acid. The results from the three gradients are superimposed and show incorporation after 10min. (■), 20min. (□) and 30min. (●).

occupied by the ribosomal subunits. This region was resolved by longer centrifuging during which most of the polyribosomes sedimented to the bottom of the gradient. Under these conditions, although some of the RNA made during inhibition sedimented with 70s ribosomes, most was in three components ('cobalt particles') that were present in almost equal amounts and whose sedimentation coefficients, estimated by interpolation, were about 44s, 33s and 23s (Fig. 1*b*). These sedimentation coefficients are used below to identify the three particles, although, as is shown, the values are not fixed parameters. The presence of these particles after incubation with cobalt chloride was not simply a consequence of a decreased rate of RNA synthesis during inhibition. When exponentially growing cells were labelled with [^{14}C]uracil for 10min. in the absence of inhibitor, RNA synthesis in this time was about half that occurring during inhibition for 30min. by Co^{2+} (Fig. 1*c*). The profiles show that the newly made RNA was then in 70s, 50s and 30s ribosomes and that components similar to the 'cobalt particles' were not apparent.

The time-course of synthesis of the 'cobalt particles' was examined. RNA made in the presence of 0·3mM-cobalt chloride was labelled with [^{14}C]-uracil as before and samples were taken from the

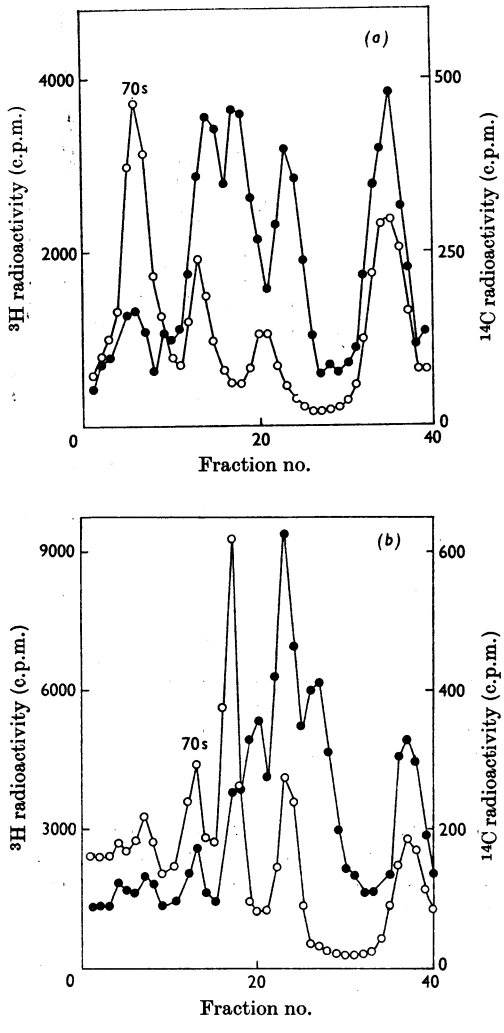

Fig. 3. Effect of different buffers on the sedimentation of 'cobalt particles'. The general procedures are described in the text. (*a*) A culture (50ml.; E_{450} 0·23) already labelled with [^{3}H]uracil was inhibited for 30min. by 0·3mM-$CoCl_2$ in the presence of 100nc of [^{14}C]uracil/ml. The cells were harvested and lysed, and a portion (0·1ml.) of the lysate was layered on to a linear sucrose density gradient made in THM buffer. (*b*) Another culture was treated similarly and a portion (0·1ml.) of the lysate layered on to an exponential sucrose density gradient made in TLMK buffer. Centrifuging was for 4hr. Fractions (2 drops) were collected for the measurement of ^{3}H radioactivity (○) and ^{14}C radioactivity (●) incorporated into material insoluble in 5% trichloroacetic acid.

culture after inhibition for 10, 20 and 30min. After lysis, portions were centrifuged in linear sucrose gradients made in THMK buffer; the three separate sedimentation profiles are superimposed in Fig. 2. The 23s and 44s components (and transfer RNA) were present after incubation for 10min. and increasing quantities were made as the inhibition continued. In contrast, the 33s particles were not detectable in the sample taken at the earliest time (10min.) although small amounts of this component would be masked by trailing of the other particles. This result implies that the syntheses of the three 'cobalt particles' are not completely co-ordinated and that the 33s material mainly accumulates under conditions that arise during, but not immediately after, inhibition by Co^{2+}.

Effect of buffer composition on the sedimentation of 'cobalt particles'. For the experiments of Figs. 1 and 2, centrifuging was in sucrose gradients made in THMK buffer. In a similar experiment in which, after inhibition for 30min. by 0·3mM-cobalt chloride, a lysate of inhibited cells was centrifuged in a gradient made in THM buffer (this solution lacks the 100mM-potassium chloride present in THMK buffer), the sedimentation characteristics of the 44s and 33s 'cobalt particles' were somewhat different (Fig. 3*a*). The 44s component sedimented to a region close to that occupied by the prelabelled 50s ribosomes; the 33s material also sedimented further by comparison with the 'marker' ribosomal subunits and was now well resolved from 30s ribosomes. The sedimentation coefficients of these two particles, estimated by interpolation and on the assumption that those of the ribosomes themselves do not alter in the different buffers, were 48s and 39s respectively.

The sedimentation properties of the 'cobalt particles' were also affected when the concentration of Mg^{2+} in the buffer was decreased. When another lysate was centrifuged in a gradient made with TLMK buffer (which contains 100mM-potassium chloride and 2mM-Mg^{2+}), there was considerable, but not complete, dissociation of polyribosomes and 70s ribosomes to ribosomal subunits (Fig. 3*b*). The 33s 'cobalt particles' were not resolved from the few 30s ribosomes formed during inhibition; on the same assumptions as above, the 44s and 23s particles now had sedimentation coefficients of about 39s and 20s respectively.

Effect of ribonuclease on 'cobalt particles'. The sedimentation profiles of the 'cobalt particles' in different buffers show that they are distinct from the 50s and 30s ribosomal subunits. Moreover, although Figs. 1(*b*) and 3(*a*) reveal that some complete 70s ribosomes are formed during inhibition, the low specific radioactivity (^{14}C radioactivity/^{3}H radioactivity) of this material suggests that, in buffers containing 10 mM-Mg^{2+}, radioactivity from newly synthesized 50s and 30s subunits does not contribute to any great extent to the regions of the gradient occupied by the 'cobalt

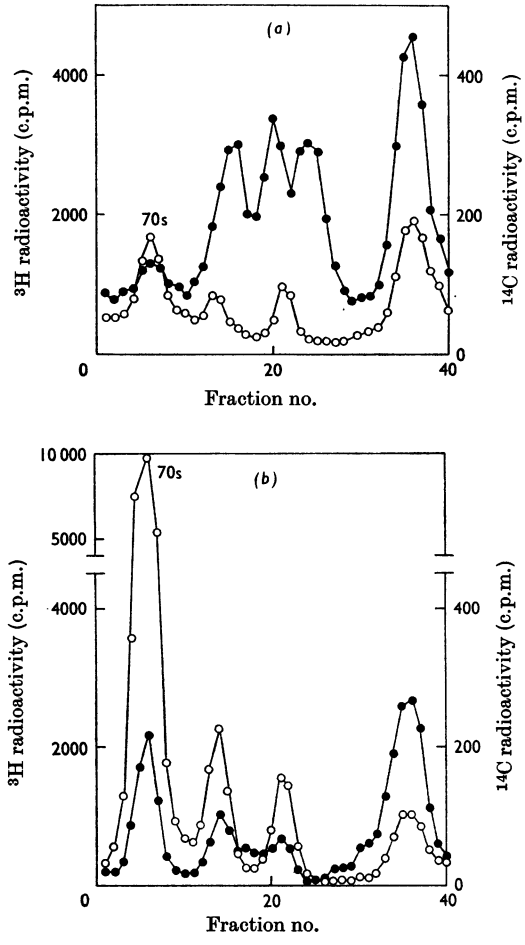

Fig. 4. Effect of ribonuclease on 'cobalt particles'. The general procedures are described in the text. A culture (50ml ; E_{450} 0·22) already labelled with [^{3}H]uracil was incubated with 0·3mM-$CoCl_2$ for 30min. in the presence of 130nc of [^{14}C]uracil/ml. The cells were collected and lysed. (*a*) A portion (0·1ml.) of the lysate was layered on to a linear sucrose density gradient made in THMK buffer. (*b*) Another portion (0·1ml.) was incubated with 10μg. of ribonuclease/ml. at room temperature for 20min. before layering on to a similar gradient. Centrifuging was for 4hr. Fractions (2 drops) were collected for the measurement of ^{3}H radioactivity (○) and ^{14}C radioactivity (●) incorporated into material insoluble in 5% trichloroacetic acid.

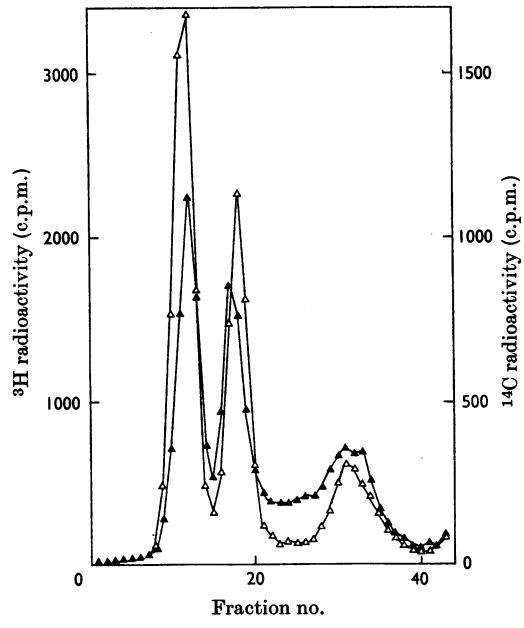

Fig. 5. Synthesis of RNA during inhibition by $CoCl_2$. The general procedures are described in the text. A culture (50ml.; E_{450} 0·23) already labelled with [^{3}H]uracil was incubated with 130nc of [^{14}C]uracil/ml. during inhibition for 30min. by 0·3mM-$CoCl_2$. The cells were harvested, lysed and treated with sodium dodecyl sulphate to liberate RNA. A portion (0·1ml.) of the lysate was layered on to a linear sucrose density gradient made in acetate buffer. Centrifuging was for 8hr. Fractions (2 drops) were collected for the measurement of ^{3}H radioactivity (△) and ^{14}C radioactivity (▲) incorporated into material insoluble in 5% trichloroacetic acid.

particles'. These suggestions are confirmed by an experiment in which cells were lysed after inhibition by cobalt chloride for 30min. A portion of the lysate was centrifuged, for a time (4hr.) sufficient to deposit the polyribosomes, in a gradient made in THMK buffer (Fig. 4*a*); another portion was treated with 10μg. of ribonuclease/ml. for 20min. at room temperature and then centrifuged (Fig. 4*b*). A comparison of the two profiles shows that the 'cobalt particles' have been almost completely degraded by the ribonuclease although the ribosomes remain intact. The large increase in the number of 70s ribosomes after ribonuclease treatment is a result of the degradation of polyribosomes brought about by the enzyme.

Synthesis of RNA during inhibition by cobalt chloride. The nature of the RNA made during inhibition by Co^{2+} was first examined. For this, a culture of exponentially growing cells labelled with [^{3}H]uracil was inhibited for 30min. by 0·3mM-cobalt chloride in the presence of [^{14}C]uracil. After lysis, sodium dodecyl sulphate was added to liberate RNA and the preparation was sedimented through a sucrose gradient made in acetate buffer (Fig. 5). During inhibition by Co^{2+}, species corresponding to transfer RNA and ribosomal RNA were synthesized. However, the profile shows that the

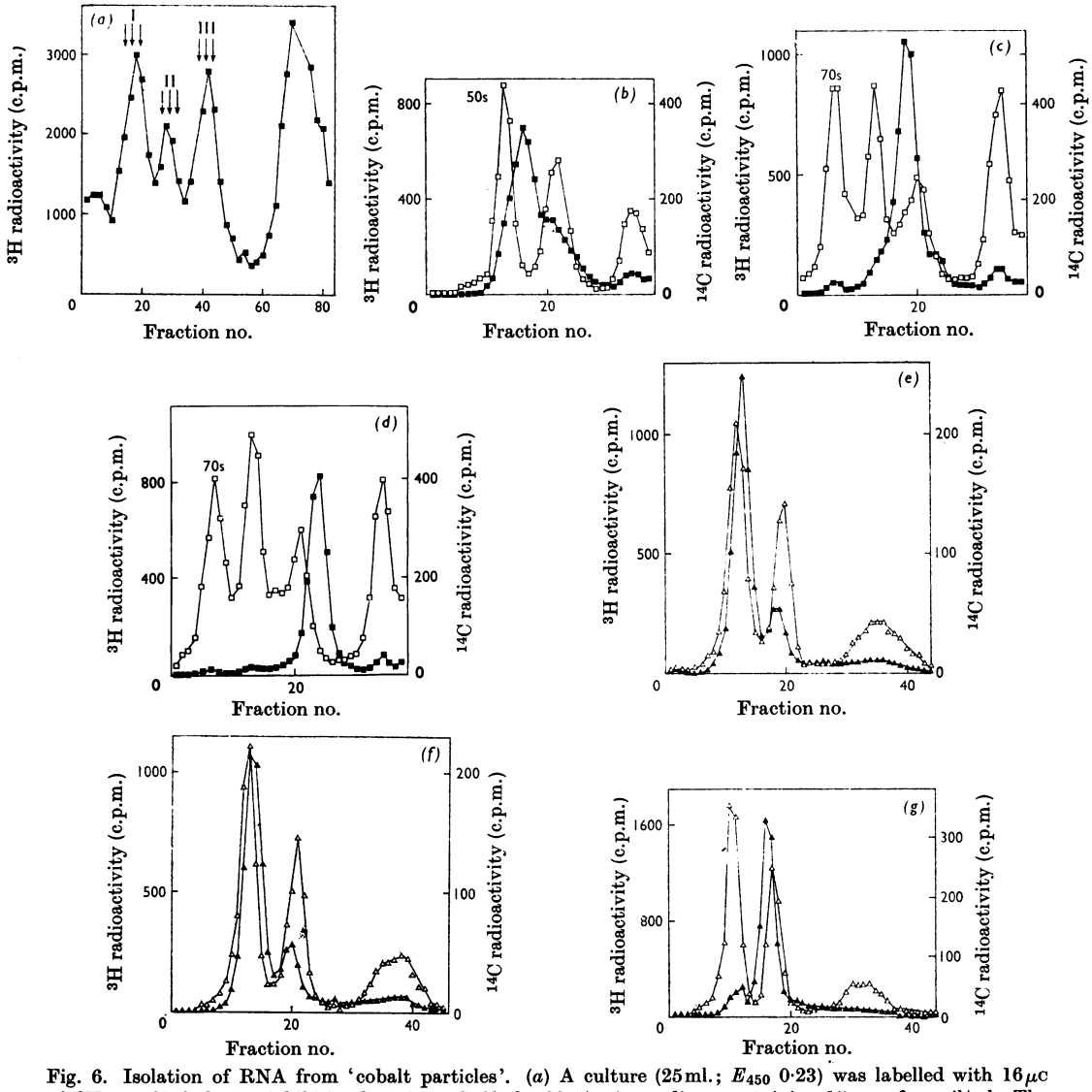

Fig. 6. Isolation of RNA from 'cobalt particles'. (*a*) A culture (25 ml.; E_{450} 0·23) was labelled with 16 μc of [^{3}H]uracil/ml. during inhibition by 0·3 mM-$CoCl_2$ for 30 min. in medium containing 15 μg. of uracil/ml. The cells were harvested and lysed. A portion (0·2 ml.) of the lysate was layered on to a linear sucrose density gradient made in THM buffer. Centrifuging was for 5 hr. About 250 drops were collected in alternate 5-drop and 1-drop fractions. Radioactivity incorporated into material insoluble in 5% trichloroacetic acid (■) was assayed in the 1-drop fractions; the 5-drop fractions indicated were pooled separately (I, II and III) and used as a source of the three 'cobalt particles'. Another culture (50 ml.; E_{450} 0·02) was grown to E_{450} 0·20 in the presence of 40 nc of [^{14}C]uracil/ml. without added carrier uracil. The cells were harvested, lysed and the lysate was diluted with 9 vol. of TM buffer. This preparation (referred to as 'Z' below) was used to supply ribosome and RNA markers for the other gradients. (*b*) Pool I (0·1 ml.) was mixed with TM buffer (0·1 ml.) and Z (40 μl.). (*c*) and (*d*) Pools II and III (0·1 ml. of each) were each mixed with THM buffer (0·1 ml.) and Z (10 μl.). The mixtures were layered on to linear sucrose density gradients made in TM buffer (*b*) or THM buffer (*c* and *d*). Centrifuging was for 4 hr. Fractions (2 drops) were assayed for ^{14}C radioactivity (□) and ^{3}H radioactivity (■) incorporated into material insoluble in 5% trichloroacetic acid. For (*e*), (*f*) and (*g*), 0·1 ml. of pools I, II and III were each mixed with THM buffer (0·1 ml.) and Z (10 μl.) and then treated with sodium dodecyl sulphate. Each mixture was layered on to a linear sucrose density gradient made in acetate buffer. Centrifuging was for 8 hr. Fractions (2 drops) were assayed for ^{14}C radioactivity (△) and ^{3}H radioactivity (▲) incorporated into material insoluble in 5% trichloroacetic acid.

specific radioactivity (^{14}C radioactivity/^{3}H radioactivity) of the newly made 23s RNA component is greater in the fractions nearer the top of the gradient. This suggests that this species of RNA has sedimented a little more slowly than the 23s RNA derived from the ^{3}H-labelled ribosomes. Conversely, the 16s RNA made during inhibition by Co^{2+} has sedimented slightly further in the gradient than the corresponding 'marker'.

Since a large proportion of the RNA made during inhibition has nearly the same sedimentation characteristics as ribosomal RNA, it is likely that these species are contained in the three kinds of 'cobalt particle'. The experiment illustrated in Fig. 6 clarifies this. The RNA made during inhibition by Co^{2+} was labelled with [^{3}H]uracil. Lysed cells were centrifuged in a sucrose density gradient in THM buffer and the peak fractions indicated on the diagram were pooled separately and used as a source of the 44s, 33s and 23s components respectively. The pooled materials were mixed with a lysate prepared from cells that had incorporated [^{14}C]uracil into stable forms of RNA in the absence of inhibitor. Centrifuging (Figs. 6*b*, 6*c* and 6*d*) showed that the three 'cobalt particles' prepared by this treatment were largely uncontaminated by each other and by ribosomal subunits. Further portions of the pooled fractions were mixed with the ^{14}C-labelled lysate and treated with sodium dodecyl sulphate. Density-gradient analyses (Figs. 6*e*, 6*f* and 6*g*) showed that the 23s particles contained a species of RNA that sedimented a little further than the corresponding 16s ribosomal-RNA marker. Both the 33s and 44s particles, on the other hand, had RNA whose sedimentation coefficients under these conditions was a little less than that of 23s ribosomal RNA.

Non-identity of 'cobalt particles' and 'chloramphenicol particles'. When *E. coli* is incubated with chloramphenicol under conditions in which there is little, if any, synthesis of protein, RNA continues to be made. A large proportion of this RNA is in two 'chloramphenicol particles'; the RNA isolated from these particles has sedimentation coefficients slightly different from those of the two species of RNA from ribosomes (Dubin & Elkort, 1964; Iwabuchi, Kono, Oumi & Osawa, 1965). Values reported for the sedimentation coefficients of the 'chloramphenicol particles' themselves (25s and 18s; Osawa, 1968) are not very different from those of the slower sedimenting 'cobalt particles' in low-Mg^{2+} buffers. However, the experiment shown in Fig. 7 suggests that 'cobalt particles' and 'chloramphenicol particles' are distinct entities. Additional reasons for this view are considered below.

A culture was grown exponentially in medium containing [^{32}P]phosphate to label stable forms of RNA. One portion was then incubated with 0·3mM-cobalt chloride for 30min. in the presence of [^{3}H]uracil and another for the same time with 100μg. of chloramphenicol/ml. and [^{14}C]uracil. The two batches of cells were then lysed separately. Portions of the lysates were mixed and centrifuged through a sucrose density gradient made in TM buffer. The ^{32}P radioactivity of the different fractions is not shown in Fig. 7, but the peak fractions corresponding to the 50s and 30s ribosomal subunits are indicated. There are small but distinct differences in the sedimentation properties of the materials accumulated in the presence of Co^{2+} and chloramphenicol; the 'cobalt particles' sedimented further in the gradient than the 'chloramphenicol particles'.

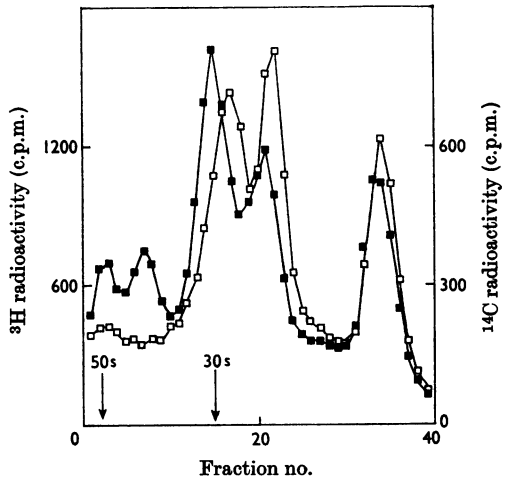

Fig. 7. Comparison of 'cobalt particles' and 'chloramphenicol particles'. The general procedures are described in the text. A culture was grown for about two generations in a 'low-phosphate' medium containing 15μg. of Na_2HPO_4/ml. and 40nc of [^{32}P]phosphate/ml. and then diluted to E_{450} 0·02 with 25vol. of normal medium containing 15μg. of uracil/ml. After growth to E_{450} 0·24, portions (50ml.) were incubated for 30min. with: (*a*) 0·3mM-$CoCl_2$ and 1·3μc of [^{3}H]uracil/ml.; (*b*) 100μg. of chloramphenicol/ml. and 270nc of [^{14}C]uracil/ml. The cells were harvested and lysed. Portions (50μl.) of each lysate were mixed and layered on to a linear sucrose density gradient made in TM buffer. Fractions (2 drops) were collected after centrifuging for 4hr. for the measurement of ^{3}H radioactivity (■), ^{14}C radioactivity (□) and ^{32}P radioactivity incorporated into material insoluble in 5% trichloroacetic acid. The ^{32}P radioactivities are not shown but peak fractions containing 50s and 30s ribosomes are indicated.

Stability of polyribosomes during inhibition by cobalt chloride. An appreciable number of ribosomes are still present in polyribosomes after inhibition for 30min. by Co^{2+} (Fig. 1). Polyribosomes are

Table 2. *Effects of cobalt chloride and chloramphenicol on polyribosomes*

A culture of *E. coli* (E_{450} 0·23) labelled with [^{3}H]uracil was divided into three portions (each of 100ml.) One was allowed to continue exponential growth and the others were incubated with $CoCl_2$ (0·3mM) and chloramphenicol (100μg./ml.) respectively. At intervals, samples (25ml.) were taken, the cells were harvested and lysed, and a portion (0·1ml.) of each lysate was layered on to an exponential sucrose gradient made in THMK buffer. After centrifuging for 45min., fractions of 2 drops were collected for the assay of radioactive material insoluble in 5% trichloroacetic acid. From the sedimentation profiles (which were similar to that given by the ^{3}H radioactivity in Fig. 1*a*) the radioactivities in the fractions containing the ribosomes (70s, 50s and 30s) and polyribosomes were summed. Results are expressed as the ratio [total radioactivity (c.p.m.) of fractions containing polyribosomes] × 100/[total radioactivity (c.p.m.) of fractions containing ribosomes and polyribosomes].

	Percentage of ribosomes as polyribosomes		
Time (min.)	Exponential growth	Inhibition by $CoCl_2$ (0·3mM)	Inhibition by chloramphenicol (100μg./ml.)
0	56	57	82
15	56	78	62
30	59	69	56
60	60	68	30

also known to persist during the inhibition of protein synthesis by chloramphenicol (Flessel, 1968; Gurgo, Apirion & Schlessinger, 1969). The effects of cobalt chloride and chloramphenicol on the proportion of the ribosomes in polyribosomes at stages throughout inhibition were compared (Table 2).

An exponentially growing culture in which [^{3}H]uracil had been incorporated into stable forms of RNA was divided into three portions. One portion was incubated with cobalt chloride (0·3mM) and one with chloramphenicol (100μg./ml.) for 1hr.; the other was allowed to continue exponential growth. At intervals, samples were lysed and centrifuged through exponential sucrose density gradients made in THMK buffer. The percentage of the ribosomes present in the polyribosomes was calculated from the radioactivities of the fractions. Chloramphenicol produced an immediate response; 82% of the ribosomes were in polyribosomes in cells harvested immediately after chloramphenicol addition (Table 2). This percentage then decreased until after incubation for 1hr. only 30% of the ribosomes were in polyribosomal material. The action of cobalt chloride was somewhat different. There was no immediate response to the addition of the inhibitor, but after incubation for 15min. 78% of the ribosomes were in polyribosomes, a value similar to that found immediately after the addition of chloramphenicol. Although the percentage subsequently decreased somewhat, the value after inhibition for 1hr. (68%) was still greater than the average value (58%) found for untreated cells.

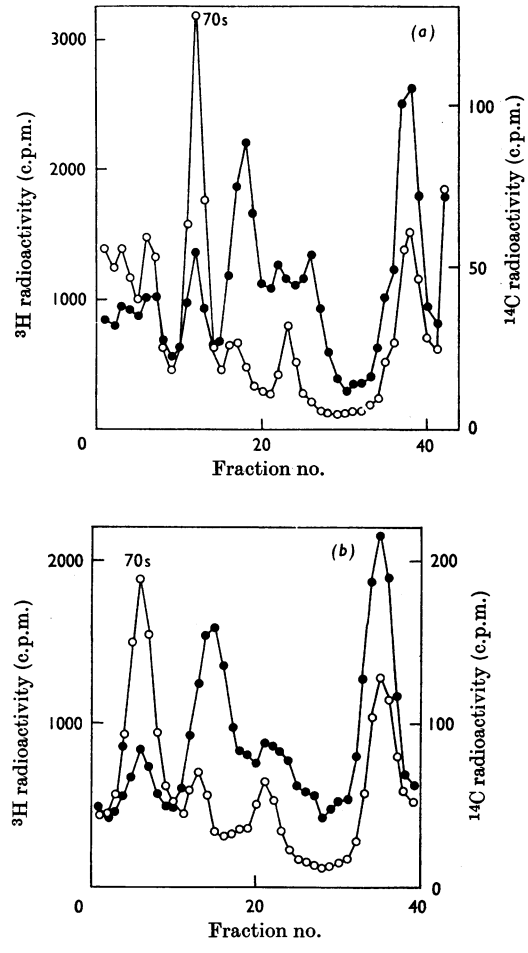

Fig. 8. Inhibition by 0·6mM- and 1mM-$CoCl_2$. The general procedures are described in the text. (*a*) A culture (50ml.; E_{450} 0·23) already labelled with [^{3}H]uracil was inhibited by 0·6mM-$CoCl_2$ for 30min. in the presence of 100nc of [^{14}C]uracil/ml. The cells were collected and lysed, and a portion (0·1ml.) of the lysate was layered on to an exponential sucrose density gradient made in THMK buffer. (*b*) Another culture (50ml.; E_{450} 0·23) already labelled with [^{3}H]uracil was inhibited by 1mM-$CoCl_2$ for 15min. in the presence of 270nc of [^{14}C]uracil/ml. The cells were harvested and lysed, and a portion (0·1ml.) of the lysate was layered on to a linear sucrose density gradient made in THMK buffer. Fractions (2 drops), collected from each gradient after centrifuging for 4hr., were assayed for ^{3}H radioactivity (○) and ^{14}C radioactivity (●) incorporated into material insoluble in 5% trichloroacetic acid.

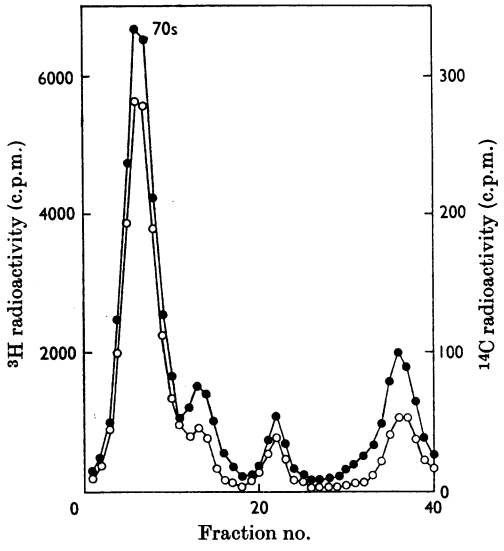

Fig. 9. Inhibition by $NiCl_2$. The general procedures are described in the text. A culture (50ml.; E_{450} 0·25) already labelled with [^{3}H]uracil was inhibited by 0·06mM-$NiCl_2$ for 30min. in the presence of 130nc of [^{14}C]uracil/ml. The cells were collected and lysed, and a portion (0·1ml.) of the lysate was layered on to a linear sucrose density gradient made in THMK buffer. Fractions (2 drops), collected after centrifuging for 4hr., were assayed for ^{3}H radioactivity (○) and ^{14}C radioactivity (●) incorporated into material insoluble in 5% trichloroacetic acid.

Inhibition by other concentrations of cobalt chloride. For the experiments described above cells that were incubated with 0·3mM-cobalt chloride for 30min. were used. Fig. 8 shows the results of experiments with 0·6mM- and 1·0mM-cobalt chloride. Some completed ribosomes were made during inhibition by 0·6mM-cobalt chloride for 30min.; however, the largest portion of the radioactivity present in the regions of the gradient occupied by the ribosomal subunits was in a species of particle whose sedimentation coefficient was about 44s; smaller amounts of 33s and 23s components are also visible in the sedimentation profile (Fig. 8*a*). Inhibition by 1mM-cobalt chloride was for 15min.; cells that were incubated for longer were found to lyse and degrade both RNA and protein. Although the RNA content of the culture increased by only 5% during the incubation, some completed ribosomes were again formed; however, a 44s particle was again evident with smaller quantities of ^{14}C radioactivity in rather ill-defined more slowly sedimenting components.

Synthesis of ribosomes during inhibition by nickel chloride. Nickel chloride inhibits the synthesis of RNA more than that of protein (Blundell & Wild, 1969). When exponentially growing cells were inhibited by 0·06mM-nickel chloride for 30min., the RNA and protein contents of the culture increased by 10% and 18% respectively (Fig. 9). Centrifuging a portion of a lysate made after inhibition showed that 70s, 50s and 30s ribosomes were made during inhibition and that particles similar to those found after incubation with cobalt chloride were not formed. Centrifuging for a shorter time (45min.) than that used in Fig. 9 gave a profile in which 20% of the ^{3}H-labelled ribosomes were present as polyribosomes; this value is much lower than those obtained for exponentially growing cells and during inhibition by Co^{2+} (Table 2).

DISCUSSION

Our results show that three species of particle accumulate during incubation of *E. coli* with cobalt chloride. These contain RNA and have sedimentation coefficients, in tris buffer containing 10mM-magnesium acetate and 100mM-potassium chloride, of about 44s, 33s and 23s. The formation of the particles is not simply a consequence of the lower rate of synthesis of RNA during inhibition, since similar entities are not observed in exponentially growing cells that have made about the same amount of RNA, nor is it a general consequence of the inhibition of bacterial growth by toxic metal ions, because cells that are inhibited by Ni^{2+} make only completed 70s, 50s and 30s ribosomes. It is likely that the appearance of the particles in lysates of cells inhibited by Co^{2+} is a consequence of the oversynthesis of RNA relative to protein that is a feature of the action of Co^{2+} but not of other inhibitory metal ions (Blundell & Wild, 1969).

Somewhat similar particles have, however, been described in bacterial cells in other circumstances. Mangiarotti, Apirion, Schlessinger & Silengo (1968) extended and clarified earlier work (reviewed by Britten, 1963) on the biosynthesis of ribosomes in *E. coli*. Two sequential precursors of 50s ribosomes were found; the sedimentation coefficients of the particles concerned were 32s and 43s. A 26s precursor to 30s ribosomes was also identified. Osawa, Otaka, Itoh & Fukui (1969) characterized precursors to 50s ribosomes from exponentially growing and pulse-labelled cells. One of these had a sedimentation coefficient of 30s in tris buffer containing 0·1mM-magnesium acetate and contained only three of the 19 proteins detected in the completed ribosome; the other had 12 proteins and a sedimentation coefficient of about 40s. These workers also detected a 26s particle, and other slower-moving components that were precursors of 30s ribosomes. In addition, 30s and 40s precursors to 50s ribosomes, similar to and probably identical with those above, accumulated during treatment

with low concentrations of chloramphenicol that inhibited but did not stop ribosome formation. It is likely that this accumulation is at least a partial consequence of the oversynthesis of RNA relative to protein known to take place during chloramphenicol inhibition. Lewandowski & Brownstein (1969) studied a 43s ribonucleoprotein component found in a strain (K106) of *E. coli* that carries a mutation suppressing streptomycin-dependence; this particle is a precursor to, and is deficient in some of the proteins of, 50s ribosomes. Strain K 106 is also characterized by a higher content of RNA relative to protein than that of the parent strain; the accumulation of a 43s precursor may here also be a consequence of oversynthesis of RNA. These considerations therefore make it likely that the particles accumulated during inhibition by Co^{2+} are ribosome precursors similar to those described above.

This possibility is made more likely by the other properties of the 'cobalt particles' that have been described. RNA isolated from the 23s 'cobalt particle' sediments slightly further in density gradients than 16s RNA from 30s ribosomes. A similar difference has been observed for the RNA extracted from precursors to 30s ribosomes obtained from cells during experiments involving both pulse-labelling and inhibition by low concentrations of chloramphenicol (Osawa, 1968; Osawa *et al.* 1969). Distinctions of this sort have been ascribed (Suzuki & Hayashi, 1964; Kono, Otaka & Osawa, 1964; Turnock & Wild, 1966) to a difference in secondary structure between the two forms of RNA perhaps as a consequence of the undermethylation (Osawa, 1968) of the RNA in ribosome precursors. The RNA from the 33s and 44s 'cobalt particles' also differs slightly in its sedimentation properties from 23s RNA from 50s ribosomes. Although the RNA species from the precursors to 50s ribosomes obtained from exponentially growing cells are undermethylated (Osawa *et al.* 1969), as may also be the RNA of the 43s particle of strain K 106 (Lewandowski & Brownstein, 1969), no differences in sedimentation properties from 23s RNA have been reported. The difference in sedimentation coefficient of the RNA from 44s and 33s 'cobalt particles' is more noticeable after their purification; a distinction between similar molecules may also depend somewhat on the buffer used in the centrifuging of solutions of RNA.

'Cobalt particles' are degraded by ribonuclease under conditions in which the ribosomes are apparently unaffected. This same phenomenon has been noted both for the ribosome precursors of exponentially growing cells (Mangiarotti *et al.* 1968) and for the 43s ribonucleoprotein component accumulated by strain K 106 (Lewandowski & Brownstein, 1969). The RNA in the 'cobalt particles' may therefore be present in less-compact structures than completed ribosomes. This view is supported by the considerable changes in the sedimentation properties of the cobalt particles that are observed during density-gradient centrifuging in buffers of different compositions.

During inhibition of *E. coli* by chlortetracycline, puromycin or high concentrations of chloramphenicol, RNA continues to be made in the virtual absence of protein synthesis. This situation is also found when protein synthesis is abruptly halted in 'relaxed' mutants. Most of the RNA made in these circumstances is in two species of particle that usually sediment more slowly than 30s ribosomes (Dagley, White, Wild & Sykes, 1962; Dagley, Turnock & Wild, 1963; Holmes & Wild, 1965) and are now thought likely to be free ribosomal RNA with which non-specific proteins can easily become associated during isolation procedures (Yoshida & Osawa, 1968; Schleif, 1968). Although these particles also contain undermethylated ribosomal RNA and are sensitive to ribonuclease, it seems likely that the 'cobalt particles' are distinct from them. The experiment of Fig. 7 shows that there are small but distinct differences in the sedimentation properties of 'chloramphenicol particles' and the two slower-moving 'cobalt particles' when lysates of cells inhibited independently with chloramphenicol and Co^{2+} are mixed and centrifuged through gradients made in a low-Mg^{2+} (TM) buffer. A comparison with this buffer was necessary because, under our conditions of lysis, chloramphenicol particles are not extracted well in the other buffers used (which contain higher concentrations of Mg^{2+}). Nomura & Watson (1959) reported a sedimentation coefficient of 31s for the faster-moving 'chloramphenicol particle' (which contains undermethylated 23s RNA; Osawa, 1968) in THM buffer; under these conditions, the sedimentation coefficients of the equivalent 'cobalt particles' are about 39s and 48s. This large difference again indicates that 'cobalt particles' and 'chloramphenicol particles' are distinct.

Thus our results suggest, but do not prove, that the three 'cobalt particles' are closely related to normal ribosome precursors. An examination of, for example, their protein contents and fate when cells recover from the inhibition caused by Co^{2+} will help to elucidate their nature.

Polyribosomes persist during inhibition of *E. coli* by Co^{2+} but are present in greatly decreased amounts after a comparable inhibition of protein synthesis by Ni^{2+}. This action of Co^{2+} is similar to, but not as rapid as, that of chloramphenicol. The latter causes an immediate increase in the percentage of ribosomes in polyribosomes, but it is not clear whether this is a result of the preservation of polyribosomes that would otherwise be lost

during the isolation procedures (Mangiarotti & Schlessinger, 1966) or a consequence of the continued synthesis of messenger RNA during the abrupt and virtually complete inhibition of protein synthesis that chloramphenicol causes. Although newly formed messenger RNA is reported to enter polyribosomes for several hours during chloramphenicol inhibition at a rate comparable with that in control cells (Gurgo *et al.* 1969), Flessel (1968) observed a decrease in polyribosome content during continued inhibition by chloramphenicol. The precise effects of Co^{2+} on ribosome function remain to be elucidated, but the persistence of polyribosomes provides support for our suggestion (Blundell & Wild, 1969) that inhibition by Co^{2+} involves some specialized effects on the protein-synthesizing apparatus similar to the actions of antibiotics whose primary effects are on protein synthesis.

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