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Altered Ribosomes after Inhibition of *Escherichia coli* by Rifampicin

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Addition of rifampicin to growing cells of *Escherichia coli* affected the ribosomes. The polyribosomes first decayed to 70S ribosomes. These later dissociated to particles distinct from ribosomal subunits. The altered ribosomes sedimented more slowly than the corresponding subunits and had lost some protein; their ribosomal RNA was intact, but they were more susceptible to degradation by ribonuclease than normal ribosomes. The addition of rifampicin to preparations of lysed cells caused no detectable changes in the ribosome fraction.

The inhibition of the growth of *Escherichia coli* by rifampicin is mediated by an effect of the antibiotic on DNA-dependent RNA polymerase. The drug binds very firmly to the isolated enzyme (Neuhoff, Schill & Sternbach, 1970) in such a way that the synthesis of new chains of RNA is prevented, although growing chains present at the time the inhibitor is added can be completed (Sippel & Hartmann, 1968). Mutants resistant to rifampicin have a RNA polymerase that, *in vitro*, is much less affected by the drug (Wehrli, Knusel & Staehelin, 1968).

In the present paper we report that incubation of *E. coli* with rifampicin eventually causes marked changes to the ribosomes, which are altered to species that sediment more slowly than 50S and 30S ribosomal subunits. These changes are described and discussed.

EXPERIMENTAL

Growth of bacteria. *E. coli* strain M.R.E.600 (Cammack & Wade, 1965) was grown in a shaking incubator with a mean generation time of about 50min at 37°C, a tris–salts–glucose medium (Blundell & Wild, 1969*a*) being used. Cultures were grown overnight in medium containing a limiting concentration of 7μg of glucose/ml to E_{450} about 0.015; in the morning, cultures received 2mg of glucose/ml and 15min later either [5-^{3}H]uracil or [2-^{14}C]uracil, as indicated in the text. After a further 45min, 15μg of non-radioactive uracil/ml was added and the cells were allowed to grow to E_{450} 0.20–0.25 before an experiment was started. For Fig. 4 and Table 1, protein was labelled by adding 670nCi of L-[U-^{3}H]lysine/ml to the culture at the same time as 27nCi of [^{14}C]uracil/ml; 20μg of L-lysine/ml was added at E_{450} 0.20.

To label RNA with [^{32}P]phosphate, cultures were grown overnight in limiting glucose as above. In the morning, the culture was diluted into 10vol. of medium containing 2mg of glucose/ml but lacking phosphate; about 0.1μCi of [^{32}P]phosphate/ml was added. The culture was grown to E_{450} about 0.5 and the cells were harvested.

Rifampicin was added to cultures as a solution in medium containing 15μg of uracil/ml and, where appropriate, 20μg of L-lysine/ml. Autoxidation of rifampicin was prevented by adding, with the antibiotic, 20μg of ascorbic acid/ml of culture. Control experiments showed that omission of ascorbate had no effect on the production of altered ribosomes by rifampicin.

Sucrose-density-gradient analysis and radioactivity measurements. The following buffers were used, all at pH7.4: (i) 10mM-tris chloride buffer–10mM-magnesium acetate–100mM-KCl (THMK buffer); (ii) 10mM-tris chloride buffer–10mM-magnesium acetate (THM buffer); (iii) 10mM-tris chloride buffer–2mM-magnesium acetate–100mM-KCl (TLMK buffer); (iv) 10mM-tris chloride buffer–0.1mM-magnesium acetate (TM buffer).

Linear sucrose gradients (15–30%, w/w) in 5.0ml of the appropriate buffer were prepared as described by Britten & Roberts (1960). Gradients were layered with 0.1ml of a preparation of lysed cells and centrifuged at 234000$g_{av.}$ at 4°C in the SW50.1 rotor of a Spinco model L2-65B ultracentrifuge. After centrifuging, about 40 fractions were collected from the bottom of a tube on to strips of Whatman GF82 glass-fibre paper and assayed for radioactivity insoluble at 0°C in 5% (w/v) trichloroacetic acid as described by Blundell & Wild (1969*b*).

Lysis of cells. This was as described previously (Blundell & Wild, 1969*b*) and essentially the method of Godson (1967). When subsequent sucrose-density-gradient centrifugation was to be in the buffers containing 10mM-magnesium acetate, the final concentration of $MgSO_4$ in the lytic mixture was also 10mM. When centrifuging was to use TM buffer or TLMK buffer, the lytic mixtures contained respectively 4mM-$MgSO_4$ or 5mM $MgSO_4$–100mM-KCl.

Analysis of RNA. For the experiment of Fig. 3, RNA was extracted from [^{3}H]uracil-labelled altered ribosomes as follows. The two sets of fractions (each 0.5ml) from a gradient were treated separately. Each was mixed with 20μl of a lysate made from a culture (25ml) that had been labelled with 80nCi of [^{14}C]uracil/ml and grown with

carrier uracil to E_{450} 0.23. Then 25μl of 10% (w/v) sodium dodecyl sulphate was added and the mixture shaken at room temperature for 10min with 0.5vol. of phenol saturated with THM buffer. The aqueous layer was removed from the lighter phenol layer and the extraction with phenol repeated twice. A mixture was then diluted with 0.75ml of 10mM-sodium acetate buffer, pH4.6, containing 100mM-NaCl. A sample (0.1ml) was centrifuged on a linear 15–30% (w/w) sucrose gradient made in the acetate buffer.

Chemicals. Radiochemicals were from The Radiochemical Centre, Amersham, Bucks., U.K. Ribonuclease was from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K., and rifampicin from Calbiochem Ltd., London W.1, U.K.

RESULTS

Formation of altered ribosomes. A culture of *E. coli* was labelled with [^{3}H]uracil and grown with carrier uracil to E_{450} 0.23. Rifampicin was then added to a final concentration of 200μg/ml. Samples of cells were collected and lysed immediately after addition of the antibiotic and at intervals thereafter; then portions of the lysates were centrifuged in sucrose density gradients made in THMK buffer (Fig. 1). The results from the sample taken at zero time were those normally obtained with untreated cells. In these conditions about 60% of the ribosomes are in polyribosomes (Blundell & Wild, 1969*b*) and so do not appear in fractions from the gradient. After inhibition for 0.5h there was an increase in the quantity of 70S ribosomes relative to tRNA, presumably as a consequence of the inhibition of mRNA synthesis by the antibiotic. By 1h the 70S ribosomes had been replaced by three components, one of which sedimented more slowly than 30S subunits present in the earlier samples; this component was also present in the later (3h) sample. The two other new components were in fractions of the gradient between those normally occupied by 50S and 30S ribosomes; after incubation for 3h only the more slowly sedimenting of these two new species remained.

Another experiment confirmed that the components formed during rifampicin treatment were distinct from ribosomal subunits. Cells labelled with [^{14}C]uracil were harvested and lysed after incubation for 1 and 2h with 200μg of rifampicin/ml. Another lysate, made from cells labelled with [^{32}P]-phosphate, provided a reference. A portion of each ^{14}C-labelled lysate was mixed with ^{32}P-labelled lysate and then centrifuged in a gradient made in THMK buffer. The results for the two gradients are superimposed in Fig. 2 and the positions of the peaks of the ribosomal subunits from the marker are indicated. The sample taken at 1h contained two components whose sedimentation coefficients, estimated by comparing the distances through which they and the ribosomal subunits had moved, were about 47S and 25S; by 2h the 47S component had been replaced by another whose sedimentation co-

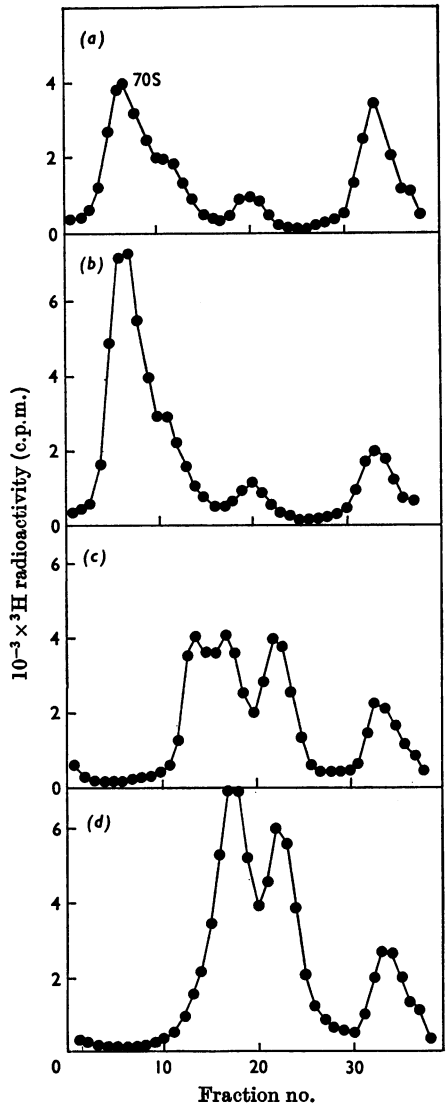

Fig. 1. Effects of rifampicin on ribosomes. A culture was labelled with 200nCi of [^{3}H]uracil/ml. A portion (100ml) was mixed with 25ml of medium containing (per ml) 15μg of uracil, 1mg of rifampicin and 100μg of ascorbic acid. Cells from samples (25ml) were collected and lysed after incubation with antibiotic for (*a*) 0h, (*b*) 0.5h, (*c*) 1h and (*d*) 3h. Portions (0.1ml) of each lysate were centrifuged for 3.5h at 124000*g* in sucrose density gradients made in THMK buffer. Fractions (2 drops) were collected for the measurement of ^{3}H radioactivity insoluble in 5% trichloroacetic acid.

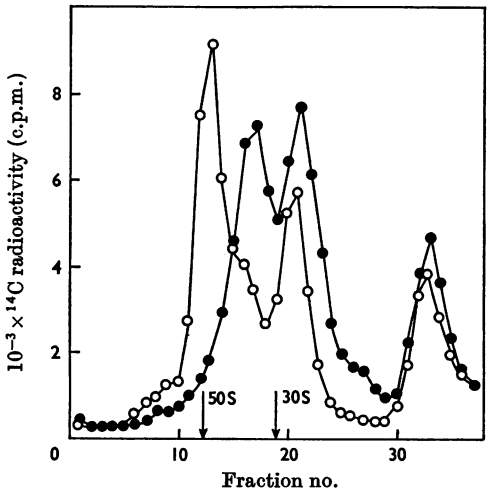

Fig. 2. Production of altered ribosomes. A culture was labelled with 100nCi of [^{14}C]uracil/ml. A portion (40ml) was then mixed with 10ml of medium containing (per ml) 15μg of uracil, 1mg of rifampicin and 100μg of ascorbic acid. Cells from samples (25ml) were collected and lysed after incubation for 1h and 2h. Then 0.1ml of each ^{14}C-labelled lysate was mixed with 2μl of ^{32}P-labelled lysate obtained from untreated cells and centrifuged for 2h in a sucrose density gradient made in THMK buffer. Fractions (2 drops) were collected for the measurement of radioactivities incorporated into material insoluble in 5% trichloroacetic acid. The ^{14}C radioactivities of the samples taken at 1h (○) and 2h (●) are superimposed. The ^{32}P radioactivities are not shown, but peak fractions containing 50S and 30S ribosomes (which were the same in both gradients) are indicated.

efficient was about 37S. The slight difference in sedimentation properties between the 47S component and 50S ribosomes was observed consistently; samples taken from cultures after incubation for 1h with rifampicin sometimes showed (in addition to the 25S species) only the 47S component and sometimes a mixture of this and the 37S species. The simple interpretation of these experiments, for which more evidence is given below, is that the material with a sedimentation coefficient of about 37S is derived from the larger ribosomal subunit through an intermediate stage. The 25S particle comes from the smaller subunit; no intermediate has here been detected.

The slower sedimentation of these altered ribosomes might be caused by decreases in the mass of the subunits due to loss of RNA and/or protein or by changes in conformation that give more open structures than the ribosomes. The experiments described below examined some of these possibilities; incubation of cultures with 200μg of rifampicin/ml was for 2 or 3h, so that only the species whose sedimentation coefficients were about 37S and 25S in THMK buffer were present in lysates of inhibited cells.

RNA in altered ribosomes. RNA was isolated from altered ribosomes by extraction with detergent and phenol. THMK buffer cannot be used in this procedure because of the insolubility of potassium dodecyl sulphate. Cells labelled with [^{14}C]uracil were incubated with rifampicin for 3h before harvesting and lysis; a portion of the lysate was mixed with lysate from uninhibited ^{32}P-labelled cells and centrifuged in a sucrose density gradient made in THM buffer (Fig. 3*a*). The two altered ribosomes were then better resolved than in THMK buffer because the sedimentation coefficient of the faster-migrating particle had increased to about 42S. This change in sedimentation properties was confirmed in other experiments, in which portions of a lysate made from inhibited cells were sedimented simultaneously through sucrose density gradients made in THMK buffer and THM buffer.

For the isolation of RNA, alternate fractions from the gradient of Fig. 3(*a*) were stored and the radioactivity of the others was measured. The fractions indicated in Fig. 3 were pooled and each pool mixed with a lysate made from uninhibited cells labelled with [^{14}C]uracil. The mixtures were deproteinized and portions centrifuged. Figs. 3(*b*) and 3(*c*) show that the slower- and faster-sedimenting altered ribosomes contained 16S and 23S rRNA and are therefore derived from 30S and 50S ribosomes respectively. Their anomalous sedimentation properties are thus not due to degradation of rRNA.

Protein content of altered ribosomes. The protein contents of the altered ribosomes were compared with those of the ribosomal subunits from which they were derived. For this, cells labelled with [^{3}H]lysine and [^{14}C]uracil were collected and lysed after incubation with 200μg of rifampicin/ml for 0, 1.5 and 3h. To compare the protein/RNA radioactivity ratios, TM and TLMK buffers were used in sucrose density gradients to dissociate the polyribosomes and 70S ribosomes in the zero-time sample. A portion of each lysate was mixed with a small portion of a 'reference' lysate made from uninhibited ^{32}P-labelled cells and then centrifuged through gradients made in TM or TLMK buffer. The profiles of the samples taken at zero time and after inhibition for 3h are shown (Figs. 4*a*–4*d*); profiles from the samples taken at 1.5h were very similar to those at 3h.

The two species of altered ribosomes were again in evidence. There was a difference in sedimentation in the two buffers similar to that noted above for THMK buffer and THM buffer. The altered ribosomes sedimented somewhat further in the buffer that lacked K^+ ions; estimated sedimentation coefficients were about 45S and 27S in TM buffer, 37S and 25S in TLMK buffer.

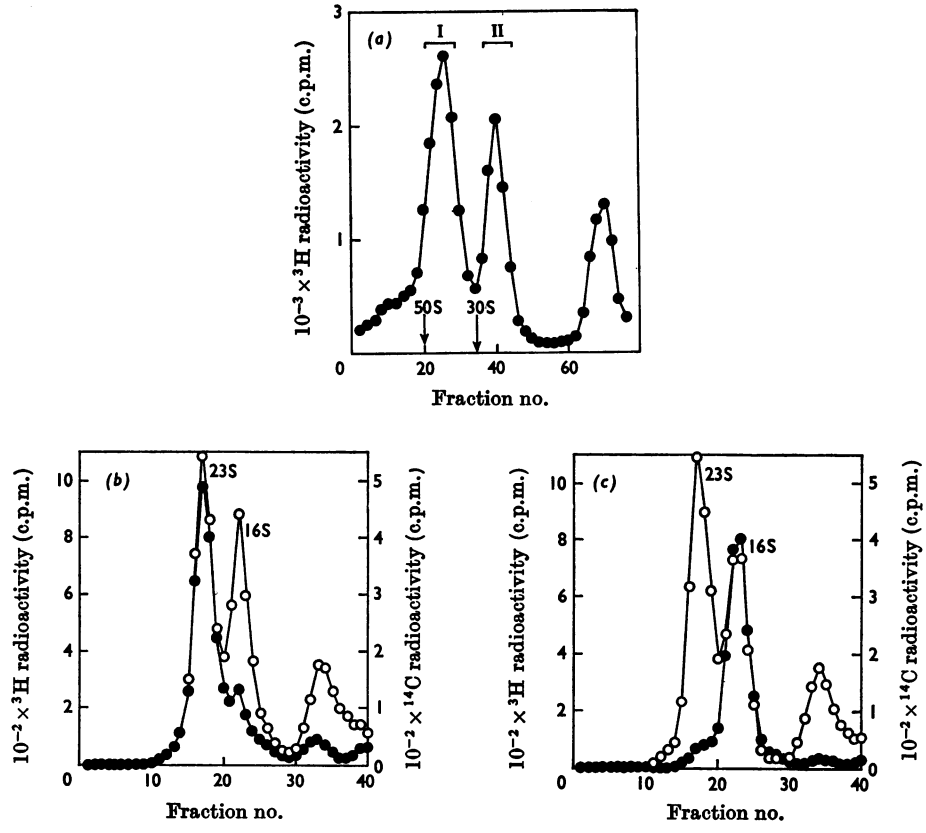

Fig. 3. RNA from altered ribosomes. (*a*) A culture was labelled with 750 nCi of [^{3}H]uracil/ml. A portion (20 ml) was then mixed with 5 ml of medium containing (per ml) 15 μg of uracil, 1 mg of rifampicin and 100 μg of ascorbic acid. After incubation for 3 h, the cells were collected and lysed. A portion (0.2 ml) of the lysate was mixed with 20 μl of a ^{32}P-labelled lysate prepared from untreated cells and centrifuged for 3 h in a sucrose density gradient made in THM buffer. About 230 drops were collected in alternate 5-drop and 1-drop fractions. The ^{3}H radioactivity incorporated into material insoluble in 5% trichloroacetic acid was assayed in the 1-drop fractions (●). The ^{32}P radioactivity is not shown, but peak fractions containing 50S and 30S ribosomes are indicated. The 5-drop fractions indicated were pooled separately (I and II) and used as a source of altered ribosomes. For (*b*) and (*c*), pools I and II were each mixed with a portion (20 μl) of a lysate made from an untreated culture that had been labelled with 80 nCi of [^{14}C]uracil/ml; preparations of RNA made from the mixtures, as detailed in the Experimental section, were centrifuged for 4.25 h in sucrose density gradients made in acetate buffer. Fractions (2 drops) were assayed for ^{3}H radioactivity (●) and ^{14}C radioactivity (○) incorporated into material insoluble in 5% trichloroacetic acid.

The protein/RNA radioactivity ratios in fractions containing the subunits and the altered ribosomes derived from them are given in Table 1. The protein/RNA radioactivity ratios for the 50S and 30S subunits (which were the same in the two buffers) were 3.4 and 3.8 respectively. Ratios for the altered ribosomes were lower; within experimental error, measurements from the 1.5 h and 3 h samples and in the two buffers gave the same results. The faster-sedimenting component had a ratio of about 3.1 and the slower 3.0. The faster-sedimenting altered ribosome therefore has about 10% less protein than 50S ribosomes and the slower-sedimenting species 20% less protein than 30S subunits.

Sensitivity of altered ribosomes to ribonuclease. Cells labelled with [^{3}H]uracil were incubated with 200 μg of rifampicin/ml for 2 h and then lysed. The lysate was mixed with another made from untreated cells labelled with [^{32}P]phosphate and then a portion of the mixture was treated with 0.05 μg of ribonuclease/ml for 5 min at room temperature. Centrifuging (Figs. 5*a* and 5*b*) showed that the ^{3}H-labelled altered ribosomes were almost completely degraded

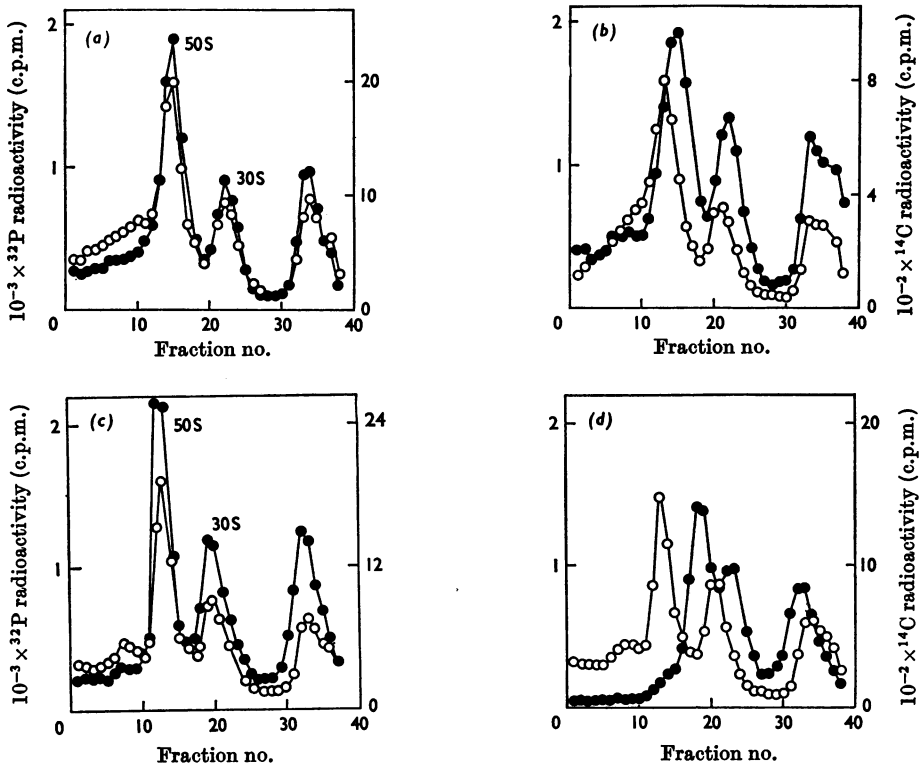

Fig. 4. Protein content of altered ribosomes. [^{3}H]Lysine and [^{14}C]uracil were used to label the protein and RNA of a culture as described in the Experimental section. A sample of culture (60ml) was then mixed with 15ml of medium containing (per ml) 15μg of uracil, 1mg of rifampicin and 100μg of ascorbic acid. The cells from portions (25ml) of the mixture were collected and lysed after incubation for 0, 1.5 and 3h. Two separate lysates were made from each mixture, one containing a final concentration of 4mM-$MgSO_4$ and the other 5mM-$MgSO_4$–100mM-KCl. Then 0.1ml of each lysate was mixed with 10μl of a lysate made, with 4mM-$MgSO_4$, from untreated cells labelled with [^{32}P]phosphate. Centrifuging was for 2.5h in sucrose density gradients made in TM or TLMK buffer. The latter buffer was used with lysates that contained KCl. Fractions (2 drops) were collected for the measurement of ^{3}H radioactivity, ^{14}C radioactivity (●) and ^{32}P radioactivity (○) incorporated into material insoluble in 5% trichloroacetic acid. The ^{3}H radioactivities are not shown, but were used to compute the values in Table 1. (*a*) TM buffer, 0h sample; (*b*) TM buffer, 3h sample; (*c*) TLMK buffer, 0h sample; (*d*) TLMK buffer, 3h sample. Profiles obtained from the samples taken at 1.5h (see Table 1) were very similar to those at 3h.

Table 1. *Protein content of altered ribosomes*

From the gradients of lysates made from cells after inhibition by 200μg of rifampicin/ml for 0, 1.5 and 3h (Fig. 4) the [^{3}H]protein/[^{14}C]RNA radioactivity ratios were computed in fractions containing ribosomal subunits or altered ribosomes. A minimum of 5000 counts of ^{14}C radioactivity was recorded in the fractions used. Each value below is the mean of the ratios for the three fractions comprising the peaks of radioactivity of the components indicated. F and S refer to the faster (F) and more slowly (S) sedimenting altered ribosomes produced during inhibition by rifampicin.

Time of sample (h)	Component	^{3}H/^{14}C radioactivity ratio	
		TM buffer	TLMK buffer
0	50S	3.45	3.44
	30S	3.86	3.76
1.5	F	3.15	3.06
	S	3.08	2.82
3	F	3.26	3.03
	S	3.02	2.91

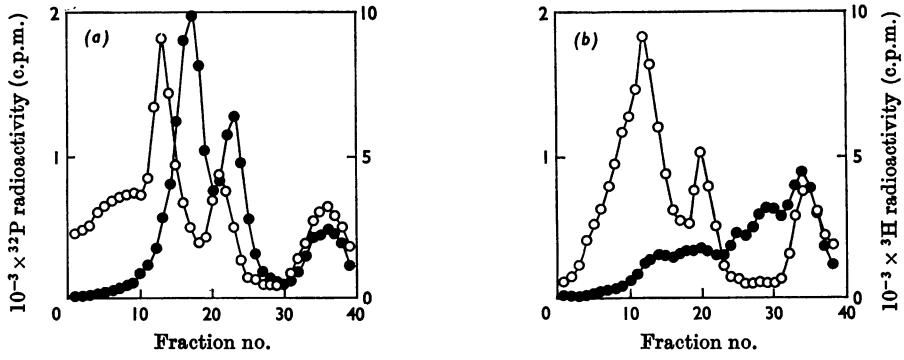

Fig. 5. Effect of ribonuclease on altered ribosomes. A culture was labelled with 500nCi of [^{3}H]uracil/ml. A portion (40ml) was mixed with 10ml of medium containing (per ml) 15μg of uracil, 1mg of rifampicin and 100μg of ascorbic acid. After incubation for 2h, cells from a sample (25ml) were collected and lysed in the presence of a final concentration of 4mM-$MgSO_4$. Then lysate (0.5ml) was mixed with TM buffer (0.6ml) and 0.1ml of a lysate made from untreated cells grown with [^{32}P]phosphate. (*a*) A portion (0.1ml) of the mixture was layered on a sucrose density gradient made in TM buffer. (*b*) Another portion (0.1ml) was incubated with 0.05μg of pancreatic ribonuclease/ml at room temperature for 5min before being layered on a similar density gradient. Centrifuging was for 2.5h. Fractions (2 drops) were collected for the measurement of ^{3}H radioactivity (●) and ^{32}P radioactivity (○) incorporated into material insoluble in 5% trichloroacetic acid.

by this treatment whereas the ^{32}P-labelled ribosomes remained apparently intact.

Effect of other concentrations of rifampicin. The experiments above used a concentration of rifampicin (200μg/ml) required to stop RNA synthesis in *E. coli* M.R.E. 600 within 1min at 37°C. However, lower concentrations of rifampicin were sufficient to affect the ribosomes. Fig. 6 shows an experiment for which lysates, made from cells after incubation with different concentrations of rifampicin for 2h, were centrifuged through gradients made in THMK buffer. The 47S and 25S components were present when the concentration of the antibiotic was 5 or 10μg/ml; at higher concentrations (20μg/ml and above) the 37S and 25S components were formed.

Action of rifampicin in vitro. A number of attempts were made to show an effect of rifampicin on ribosomes *in vitro*. For these, cells labelled with radioactive uracil were lysed, diluted with THM buffer or THMK buffer and incubated with and without added rifampicin (200μg/ml) at 37°C for up to 1.5h. They were then mixed with an unincubated lysate made from ^{32}P-labelled cells and centrifuged in sucrose density gradients. Incubation with or without rifampicin caused no significant changes in the sedimentation profiles.

DISCUSSION

Inhibition of *E. coli* M.R.E. 600 by 200μg of rifampicin/ml produced a series of changes in the sedimentation properties of the ribosomes. First, the polyribosomes dissociated to 70S ribosomes probably as a consequence of depletion of mRNA after inhibition of RNA synthesis by rifampicin, in which case 'run-off' ribosomes would be produced. It is arguable (Mangiarotti & Schlessinger, 1966; Kohler, Ron & Davis, 1968) whether these should be expected to remain as 70S ribosomes or dissociate into subunits. However, we have examined (M. R. Blundell & D. G. Wild, unpublished work) the sedimentation in THMK buffer of ribosomes from cells whose mRNA has been depleted by glucose starvation; as found by Kohler *et al.* (1968), the dissociation of polyribosomes was accompanied by a comparable increase in the content of 70S ribosomes.

The disappearance of 70S ribosomes during continued incubation with rifampicin was therefore unexpected. The sedimentation properties of the components formed from them suggest that changes in structure may be interfering with interactions that normally join the subunits. No alteration in 23S and 16S rRNA occurs, but some 10% of protein is lost from the 50S subunits and about 20% from 30S ribosomes. The sedimentation coefficients of the subunits decreased, in THMK buffer, by about 25% and 17% respectively to 37S and 25S. The loss of 10% of protein from the 50S subunit seems scarcely sufficient to account for the large change in sedimentation coefficient. It is likely that the new components have more open structures than the subunits; this might account for the variability in sedimentation properties of the component derived from 50S ribosomes and for the increased sensitivity of the altered ribosomes to ribonuclease. A somewhat similar variation in sedimentation properties has been noted for the ribonuclease-sensitive protein-deficient ribosome precursors that accumulate during

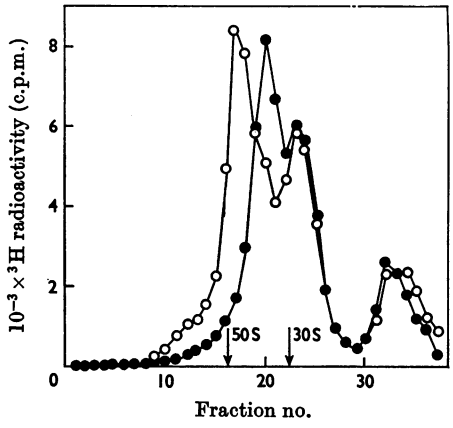

Fig. 6. Effects of different concentrations of rifampicin. A culture was labelled with 200nCi of [^{3}H]uracil/ml. Portions (each of 20ml) were mixed with medium containing (per ml) 15μg of uracil, 1mg of rifampicin and 100μg of ascorbic acid so as to give final concentrations of antibiotic of 5, 10, 20, 50, 100 and 200μg/ml. After incubation for 2h, cells were collected and lysed. Then 0.1ml of each lysate was mixed with 50μl of a lysate made from untreated cells grown in medium containing [^{32}P]-phosphate and then centrifuged for 2h in a sucrose density gradient made in THMK buffer. Fractions (2 drops) were collected for the measurement of radioactivities incorporated into material insoluble in 5% trichloroacetic acid. The ^{3}H radioactivities of the samples that had been incubated with 10μg (○) and 20μg (●) of rifampicin/ml are superimposed. ^{32}P radioactivities are not shown, but peak fractions containing 50S and 30S ribosomes (which were the same in both density gradients) are indicated. The profiles of radioactivity of the samples that had been incubated with 20, 50, 100 and 200μg of rifampicin/ml were very similar; so were those in which 5 and 10μg of antibiotic/ml had been used.

inhibition of *E. coli* M.R.E. 600 by cobalt chloride (Blundell & Wild, 1969*b*, and unpublished work).

The mechanism by which rifampicin alters the ribosomes is obscure. It is not likely to be directly related to the rapid inhibition of RNA polymerase. However, inhibition of RNA synthesis must cause major changes in the metabolism of treated cells. Maruyama, Ono & Mizuno (1970) observed alterations in the sedimentation of ribosomes, somewhat similar to those induced by rifampicin, when cells of *E. coli* strain B from an exponentially growing culture were incubated for 3h in buffer lacking all nutrients. Related experiments examined the effects of starvation for Mg^{2+} or phosphate ions (Maruyama & Mizuno, 1970*a*,*b*); the ribosomes then broke down and no slower-sedimenting species were observed.

An alternative possibility is that rifampicin has a second activity independent of the inhibition of RNA polymerase. Such secondary effects are not uncommon among antibiotics. Although Hartmann, Honikel, Knüsel & Nuesch (1967) found that rifampicin did not inhibit the synthesis of polyphenylalanine in a cell-free system derived from *E. coli* and to which polyuridylic acid had been added, Calvori, Frontali, Leoni & Tecce (1965) reported that rifamycin M_{14} inhibited protein synthesis *in vitro* in a system derived from *Bacillus subtilis* and primed with either polyuridylic acid or high-molecular-weight RNA from *Bacillus stearothermophilus*. They suggested that the antibiotic might have a direct effect on ribosomes. Later, Calvori, Frontali, Leoni & Tecce (1967) found that rifamycin M_{14} produced no changes in the sedimentation of ribosomes when added to cell-free extracts of *B. subtilis*, a result similar to our failure to affect ribosome sedimentation patterns in *E. coli* by the addition of rifampicin *in vitro*. However, these authors also reported experiments in which rifamycin M_{14} was added to growing cultures of *B. subtilis*; after inhibition for 1 or 1.5h, extracts were prepared by grinding with alumina in THM buffer. Analytical ultracentrifugation gave schlieren diagrams showing that the proportion of 70S ribosomes in the extracts decreased somewhat after inhibition, with a corresponding increase in the ribosomal subunits present. These workers may therefore have been observing preliminary stages in the changes that we have described.

The changes in the sedimentation of ribosomes that are induced by rifampicin will probably inactivate them; inactivation may precede detectable changes in sedimentation properties. Thus care may be necessary in interpreting the results of experiments that measure the rate of protein synthesis in rifampicin-treated cells (for example to determine their content of mRNA), particularly when incubations with the antibiotic are prolonged.

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